SNPlex™ Genotyping System 48-plex

User Guide

Applied Biosystems

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Preface

How to Use This Guide

Purpose of This Guide

The Applied Biosystems *SNPlex™ Genotyping System 48-plex User Guide* provides information on how to use the SNPlex™ System kits with the Applied Biosystems 3730/3730*xl* DNA Analyzer. This document includes new information about:

- Improved protocols for 384-well and 96-well plates.
- Control (ligation probe) pool and dried gDNA plates kit, which you can use to evaluate the performance of the SNPlex System.
- Use of GeneMapper® Software v3.7 to analyze SNPlex System data.
  This version of the software provides a new clustering algorithm (Model), which raises the accuracy of scoring. The existing Rules clustering algorithm is also included.

IMPORTANT! Chapters 1 and 3 describe significant changes in the assay setup.

Audience

This guide is intended for novice and experienced SNPlex™ Genotyping System 48-plex users who perform SNPlex System assays and analyze the data using GeneMapper software.

Text Conventions

This guide uses the following conventions:

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

  Right-click the sample row, then select **View Filter > View All Runs**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The size of the column affects the run time.

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid Oracle user ID and password.

IMPORTANT! You must create a separate Sample Entry Spreadsheet for each 96-well plate.

Safety Alert Words

Safety alert words also appear in user documentation. For more information, see "Safety Alert Words" on page xvi.

How to Obtain More Information

Related Documentation

The following related documents are available:

- *SNPlex™ Genotyping System, 48-plex Assay Design and Ordering Guide* (PN 4357460) – Describes the SNPlex Genotyping Systems assay design and ordering process, the file formats to use for a successful submission, and guidelines to maximize the assay design success rate.
- *SNPlex™ Genotyping System, 48-plex Quick Reference Card* (PN 4360855)– Provides condensed procedures for using the SNPlex Genotyping System 48-plex.
- *SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide* (PN 4363143) – Assists principal investigators and laboratory staff with using the SNPlex Genotyping System 48-plex with general robotics.
- *SNPlex™ Genotyping System 48-plex Automating OLA Using the Biomek FX Getting Started Guide* (PN 4360796) – Explains how to set up the Biomek FX instrument for automating the OLA portion of the SNPlex System assay.
- *SNPlex™ Genotyping System 48-plex Automating OLA Using the TECAN Genesis RSP Getting Started Guide* (PN 4360790) – Explains how to set up the TECAN Genesis RSP instrument for automating the OLA portion of the SNPlex System assay.
- *SNPlex™ Genotyping System 48-plex Automation Guide Automating PCR Using the Tomtec Quadra 3 Getting Started Guide* (PN 4358100) – Explains how to set up the Tomtec Quadra 3 instrument for automating the post-PCR portion of the SNPlex System assay.
- *Applied Biosystems 3730/3730xl DNA Analyzers Getting Started Guide* (PN 4331468) – Provides information about using the 3730/3730xl instrument.
- *GeneMapper® Software v3.7 Online Help* – Describes the analysis software and provides procedures for common tasks.

Note: For additional documentation, see "How to Obtain Support" on page xiii.

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Safety Information

This section includes the following topics:

Safety Conventions Used in This Document

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below:

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

– Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

– Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

– Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard icons that are affixed to Applied Biosystems instruments*.

Examples

The following examples show the use of safety alert words:

IMPORTANT! You must create a separate a Sample Entry Spreadsheet for each 96-well microtiter plate.

The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.

CHEMICAL HAZARD. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

ELECTRICAL HAZARD. Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.

Chemical Safety

Chemical Hazard Warning

WARNING CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

WARNING CHEMICAL HAZARD. All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING CHEMICAL HAZARD. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

WARNING CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page xvii.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Safety

Chemical Waste Hazard

HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.

CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.

- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

General Biohazard

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

Workstation Safety

Correct ergonomic configuration of your workstation can reduce or prevent effects such as fatigue, pain, and strain. Minimize or eliminate these effects by configuring your workstation to promote neutral or relaxed working positions.

MUSCULOSKELETAL AND REPETITIVE MOTION HAZARD. These hazards are caused by potential risk factors that include but are not limited to repetitive motion, awkward posture, forceful exertion, holding static unhealthy positions, contact pressure, and other workstation environmental factors.

To minimize musculoskeletal and repetitive motion risks:

- Use equipment that comfortably supports you in neutral working positions and allows adequate accessibility to the keyboard, monitor, and mouse.
- Position the keyboard, mouse, and monitor to promote relaxed body and head postures.

Introduction 1

This chapter covers:

Product Overview

The human genome contains between 6 million and 30 million single nucleotide polymorphisms (SNPs). Geneticists estimate that 300,000 or more SNPs may be required per individual to map complex diseases, such as cancer and heart disease, in a given population.

Product Description

The SNPlex™ Genotyping System uses Applied Biosystems oligonucleotide ligation assay (OLA) combined with multiplex PCR technology to achieve allelic discrimination and target amplification. The chemistry is made possible through the use of a set of universal core reagent kits and a set of SNP-specific ligation probes.

The complete SNPlex Genotyping System contains the following components:

- **Universal SNPlex System Kits and reagents** – The SNPlex System Kits contain all the reagents needed to perform the SNPlex System Assay. "SNPlex System Kits and Reagents" on page 1-8 provides details about each kit.
- **SNP-specific ligation probes** – Applied Biosystems designs SNP-specific probes (also called assays) based on the SNPs that you specify. You can submit SNPs as IDs from common databases, such as dbSNP or the Celera Discovery System™ online platform, or as custom sequences containing your SNPs of interest. For more information about the assay design process, refer to the *SNPlex™ Genotyping System, 48-plex Assay Design and Ordering Guide* (PN 4357460).
- **Validated SNP content** – Applied Biosystems SNPbrowser™ software provides annotated locus information (generated by Celera Genomics) allowing you to design assays based on polymorphisms in human DNA samples.
- **Genotyping analysis software** – The SNPlex System software suite consists of Data Collection v2.0 or higher and GeneMapper® 3.7 software.

  The suite allows you to collect and manage raw data, process and analyze the data, and finally store and manage the processed locus, sample, and called genotype information. By working with Applied Biosystems Professional Services[1], you can add sample tracking and consumables management capabilities to your software.
- **Electophoresis instruments and consumables** – The Applied Biosystems 3730/3730*xl* DNA Analyzers (3730/3730*xl* analyzers) enable the separation and detection of SNP-specific reporter probes using standard capillary arrays, electrophoresis buffers, and polymers. A unique matrix standard allows you to spectrally calibrate your electrophoresis instruments.

1. **Informatics Software and Services**, Applied Biosystems, 3833 North First Street, San Jose, CA 95134 USA

Phone: 800.762.5467

E-mail: informatics@appliedbiosystems.com

In Europe, contact Informatics by e-mail at: InformaticsInfo@eur.appliedbiosystems.com or by fax at +44.0.1925.282.502

In Asia Pacific, contact Informatics by e-mail at: InformaticsAsiaPacific@appliedbiosystems.com by phone at +65.6896.1036, or by fax at +65.6896.2147

About SNP-Specific Probes

Each assay includes three SNP-specific ligation probes:

- Two of the probes are allele-specific oligos (ASOs). These are designed specifically for the detection of polymorphisms by having the discriminating nucleotide on the 3′ end. Each ASO probe sequence also contains one of 96 unique ZipCode™ sequences for ZipChute™ probe binding.

  In a multiplex reaction, the universal ZipCode sequences on each ASO are unique. Therefore, in a 48-plex reaction, there are 96 ASOs (two for each SNP), and 96 different ZipCode sequences.
- The third probe is a locus-specific oligo (LSO). Its sequence is common to both alleles of a given locus and anneals adjacent to the SNP site on its target DNA. Each LSO also contains a partial universal PCR-primer binding site.

  In a 48-plex reaction, there are 48 LSOs.

All 144 probes for a 48-plex reaction are shipped together as an ASO/LSO probe pool. It is this pool that confers genotyping specificity to the SNPlex System assay. All other reagents are universal and not SNP specific.

Order SNP-specific probes separately (through the myScience℠ research environment described in the *SNPlex™ Genotyping System, 48-plex Assay Design and Ordering Guide*). SNP-specific probes are not included in the SNPlex System kits.

About Universal ASO/LSO Linkers

The SNPlex System Oligonucleotide Ligation Kit includes a set of Universal ASO/LSO linkers.

- Each ASO is ligated to a universal ASO-specific linker. These linkers contain
  - A universal PCR primer sequence corresponding to the universal forward primer (UA sequence)
  - A partial cZipCode sequence

  The ASO linkers anneal to the universal ZipCode sequence of the ASO probes. In a 48-plex reaction, there are 96 different ASO linkers (one for each of the 96 ASO probes); each 48-plex utilizes the same set of 96 universal ASO linkers. Although the ASO linkers anneal to specific ZipCode sequences, they are not SNP-specific.
- One additional linker is ligated to the LSO and has a universal sequence that is compatible with all LSOs. (That is, there is only one LSO linker in a 48-plex reaction.) The sequence includes a partial binding site for a universal reverse primer.
- Each linker contains a spacer that protects a complete ligation product from exonuclease digestion.

Figure 1-1 shows the interaction between SNP-specific probes and universal linkers.

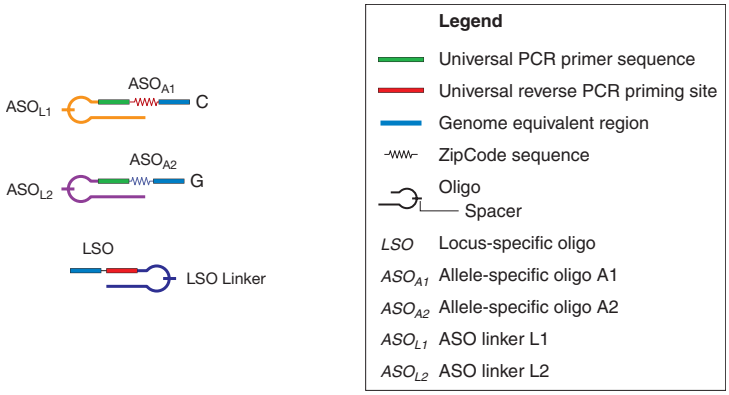


Figure 1-1 Interaction between SNP-specific probes and universal linkers

About ZipChute Probe-Based Chemistry

ZipChute™ probes are used for decoding the genotype information by functioning as reporter probes.

Each ZipChute probe has:

- A **ZipCode-binding sequence** – This sequence binds to the single-stranded cZipCode (complementary ZipCode) region of the PCR products.
- **Mobility modifiers** – Each ZipChute probe contains a different number of mobility modifiers, which enable size separation during electrophoresis.
- A **fluorescent label** – The fluorescent dye allows the 3730/3730*xl* DNA Analyzer to detect the ZipChute probe.

ZipChute probes are arranged in pairs, each pair representing both alleles of a SNP. The peaks of a pair, observed after electrophoretic separation, are used to identify the alleles of the corresponding SNP. Because each allele within a locus is represented by the same color, the SNPlex System uses both the size and color of the ZipChute probes to resolve alleles within a locus.

The SNPlex System Hybridization Kit contains a universal ZipChute mixture, which can be used for all multiplex reactions. The ZipChute probes constitute a library of reference alleles, called an allelic ladder. The master set of probes is used to normalize GeneMapper software parameters and aid in simplifying and automating allele scoring.

Figure 1-2 shows the functional parts of a ZipChute probe.

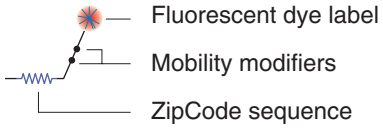


Figure 1-2 Parts of a ZipChute probe

Supported Configurations

Table 1-1 lists the supported configurations for the SNPlex 48-plex system.

Table 1-1 Supported configurations for the SNPlex 48-plex System

Instrument	# of Capillaries	Plate Type/Protocol[a]	Automation Options
3730	48	96-well	None
		384-well	None
3730*xl*	96	96-well	None
		384-well	Yes[b]

a. Chapter 3 provides the 96- and 384-well protocols for the 3730/3730*xl* analyzers.

b. Refer to the *SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide* (PN 4363143) for more information about automating the SNPlex System assay. Also see the specific robotics Getting Started Guides under "Related Documentation" on page xii and "SNPlex System Documentation" on page 1-6.

SNPlex System Documentation

Table 1-2 lists the documentation available for the SNPlex Genotyping System 48-plex.

Table 1-2 SNPlex Genotyping System, 48-plex documentation

Document Title	Part Number	Contents	Availability
SNPlex™ Genotyping System 48-plex Assay Design and Ordering Guide	4357460	• Explains how to design SNPlex System ligation probes • Provides instructions for using the Web site to order SNPlex System ligation probes • Describes the error conditions that you may encounter when designing probes	• Part of the SNPlex Genotyping System Starter Kit, 48-plex • Downloadable from the SNPlex System Web site[a] , the Documents-on-Demand Web site[b], and the myScience environment Web site[c]
SNPlex™ Genotyping System 48-plex User Guide (this document)	4360856	• Describes the SNPlex Genotyping System 48-plex • Explains how to set up the 3730 and 3730*xl* instruments for use with the SNPlex System • Contains the 96-and 384-well protocols for the 3730/3730*xl* analyzers • Provides information about analyzing SNPlex System data with GeneMapper Software v3.7 • Discusses the troubleshooting process for SNPlex System experiments	• Part of the SNPlex Genotyping System Starter Kit, 48-plex • Downloadable from the SNPlex System Web[a] site and Documents-on-Demand Web site[b]
SNPlex™ Genotyping System 48-plex Quick Reference Card	4360855	Contains short versions of the 96-and 384-well protocols for the 3730/3730*xl* analyzers, as well as summaries of troubleshooting and GeneMapper software analysis.	• Part of the SNPlex Genotyping System Starter Kit, 48-plex • Downloadable from the SNPlex System Web site[a] and Documents-on-Demand Web site[b]
SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide	4363143	• Describes options for automating the SNPlex System assay using liquid-handling instruments • Describes the laboratory set up for automation • Provides a sample automated workflow	• Part of the SNPlex Genotyping System Starter Kit, 48-plex • Downloadable from the SNPlex System Web site[a] and Documents-on-Demand Web site[b]
SNPlex™ Genotyping System 48-plex Automating OLA Using the Biomek FX Getting Started Guide	4360796	• Explains how to set up the Biomek FX instrument for automating the OLA portion of the SNPlex System assay • Provides the step-by-step automation protocols for using the Biomek FX instrument to automate the OLA portions of the SNPlex System assay	Downloadable from the SNPlex System Web site[a] and Documents-on-Demand Web site[b]

Table 1-2 SNPlex Genotyping System, 48-plex documentation *(continued)*

Document Title	Part Number	Contents	Availability
SNPlex™ Genotyping System 48-plex Automating OLA Using the TECAN Genesis RSP Getting Started Guide	4360790	• Explains how to set up the TECAN Genesis RSP instrument for automating the OLA portion of the SNPlex System assay • Provides the step-by-step automation protocols for using the TECAN Genesis RSP instrument to automate the OLA portions of the SNPlex System assay	Downloadable from the SNPlex System Web site[a] and Documents-on-Demand Web site[b]
SNPlex™ Genotyping System 48-plex Automating PCR Using the Tomtec Quadra 3 Getting Started Guide	4358100	• Explains how to set up the Tomtec Quadra 3 instrument for automating the post-PCR portion of the SNPlex System assay • Provides the step-by-step automation protocols for using the Tomtec Quadra 3 instrument to automate the post-PCR portions of the SNPlex System assay	Downloadable from the SNPlex System Web site[a] and Documents-on-Demand Web site[b]
Related Documentation			
Applied Biosystems 3730/3730xl DNA Analyzers Getting Started Guide	4331468	Provides information about using the 3730/3730*xl* instrument	Downloadable from the Documents-on-Demand Web site[b]
GeneMapper® Software v3.7 Online Help	NA	Describes the analysis software and provides procedures for common tasks	Installed with GeneMapper software

a. http://www.allsnps.com

b. http://docs.appliedbiosystems.com/search.taf/

c. http://myscience.appliedbiosystems.com/snplex/snplexStart.jsp

SNPlex System Kits and Reagents

Overview

The reagents you need to perform a SNPlex System assay are provided in six parts:

- A core reagents kit, which contains reagents required to perform the SNPlex System assay
- A starter kit, which contains additional reagents required by first-time SNPlex System users, as well as documentation
- The ligation probes
- Two sets of hybridization plates (96-well or 384-well)
- A control pool
- A dried gDNA plates kit

These reagents are described in the following section.

Kits and Reagents

Reagent Name	Part Number
SNPlex™ Genotyping System Core Reagents Kit[a], 48-plex	4362266
SNPlex™ System Assay Control Kit[a] Control DNA SNPlex™ System	4349363
SNPlex™ System Oligonucleotide Ligation Kit[a] • Universal Linkers, 48-plex SNPlex™ System • Oligonucleotide Ligation Master Mix SNPlex™ System • dATP (100X) SNPlex™ System	4362268
SNPlex™ System Purification Kit[a] • Lambda Exonuclease SNPlex™ System • Exonuclease Buffer (10X) SNPlex™ System • Exonuclease I SNPlex™ System	4349357
SNPlex™ System Amplification Kit[a] • Amplification Primers (20X) SNPlex™ System • Amplification Master Mix (2X) SNPlex™ System	4349358
Hybridization Binding Buffer SNPlex™ System	4349304
Hybridization Wash Buffer (10X) SNPlex™ System	4349301
ZipChute™ Dilution Buffer SNPlex™ System	4349306
SNPlex™ System ZipChute™ Kit, 48-plex • Denaturant SNPlex™ System • ZipChute™ Mix, 48-plex SNPlex™ System • Positive Hybridization Controls SNPlex™ System	4349361
SNPlex™ System Standards Kit, 48-plex • Sample Loading Reagent SNPlex™ System • Size Standard, 48-plex SNPlex™ System • Allelic Ladder, 48-plex SNPlex™ System	4349351

Reagent Name	Part Number
SNPlex™ System Starter Kit, 48-plex	4362267
SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD	4366107
SNPlex™ Genotyping System 48-plex User Guide	4340856
SNPlex™ Genotyping System 48-plex Quick Reference Card	4340855
SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide	4363143
SNPlex™ System 48-plex Support Files CD	4352129
SNPlex™ System Array Conditioning Kit	4352018
SNPlex™ System Control Pool, 48-plex	4362635
SNPlex™ System Dried gDNA Plates	4362637
DS-40 Spectral Calibration Standard Kit (Dye Set S)	4349365
SNPlex™ System Control Pool Kit • Control Pool, 48-plex SNPlex™ System • SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD	4362639
SNPlex™ System Dried gDNA Plates Kit • Dried gDNA Plate SNPlex™ System • SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD	4366135
SNPlex™ System Hybridization Plates, 384-well (5 plates)	4349369
SNPlex™ System Hybridization Plates, 96-well (5 plates)	4357279
SNPlex™ System Hybridization Plates, 96-well (10 plates)	4362933
SNPlex™ System Ligation Probes	4346978

a. Each SNPlex System kit provides sufficient reagent to perform 5,000 reactions. If you do not expect to consume all the reagents in a kit in a single use, Applied Biosystems recommends that you aliquot the reagents to minimize repeated freeze-thaw cycles.

Ordering SNPlex System Kits and Reagents

First-time Orders

The first time you order SNPlex System reagents, you must order

- Ligation probes
- A starter kit
- A core reagents kit
- A set of hybridization plates, either 96-well or 384-well depending on your experiment
- A control pool
- A dried gDNA plates kit

Subsequent Orders

As you consume the reagents, you can order ligation probes, core reagents, hybridization plates, control pools, and dried gDNA plates kits as needed.

Note: You can order components of the core reagents kit individually (using the individual kit part numbers instead of the core reagent kit part number).

Required Non-Kit Materials

Equipment and Consumables

This is a list of all of the required materials not provided in the kits.

Table 1-3 Required equipment and consumables

Item		Vendor	Part Number
Applied Biosystems 3730/3730*xl* DNA Analyzer		See your Applied Biosystems representative for information.	
Consumables	POP-7™ Performance Optimized Polymer	Applied Biosystems	4335615
	DS-40 Spectral Calibration Standard Kit (Dye Set S)	Applied Biosystems	4349365
	10× Running Buffer with EDTA	Applied Biosystems	4335613
	36-cm 48-capillary array (3730 instrument)	Applied Biosystems	4331247
	36-cm 96-capillary array (3730*xl* instrument)	Applied Biosystems	4331244
GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module or GeneAmp® PCR System 9700 Dual 96-Well Sample Block Module		See your Applied Biosystems representative for information.	
Reaction Plates	MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems	N8010560
	ABI PRISM® 384-Well Clear Optical Reaction Plate, with Barcode, 50 plates	Applied Biosystems	4309849
	ABI PRISM® 384-Well Optical Reaction Plate with Barcode, 500 plates	Applied Biosystems	4326270
	384-Well Plate Base (heat-sealed)	Applied Biosystems	4334877
	384-Well Plate Retainer (septa-sealed)	Applied Biosystems	4334868
	96-Well and 384-Well Plate Retainer (heat-sealed)	Applied Biosystems	4334865
	Heat Seal Film	Applied Biosystems	4337570
	96-Well Sample Plates w/barcode	Applied Biosystems	4306737
	96-Well Plate Septa	Applied Biosystems	4315933

Table 1-3 Required equipment and consumables *(continued)*

Item		Vendor	Part Number
Reaction Plates (*continued*)	96-Well Plate Base (septa-sealed)	Applied Biosystems	4334873
	96-Well Plate Base (heat-sealed)	Applied Biosystems	4334875
	96-Well Plate Retainer (septa-sealed)	Applied Biosystems	4334869
	384-Well Plate Septa	Applied Biosystems	4315934
	384-Well Plate Base (septa-sealed)	Applied Biosystems	4334874
Reaction Plate Covers[a]	MicroAmp® Full 96-Well Plate Cover	Applied Biosystems	N8010550
	ABI PRISM® Optical Cover Compression Pad	Applied Biosystems	4312639
• Heat seals and sealers	Easy-Peel 610 meter roll	ABGene	AB-3739
	Easy-Peel individual sheets	ABGene	AB-0745
	UNISEAL AL	Whatman	7704-0002
	Plate Sealer, ALPS 300™	ABGene	AB-0950
	Thermo-Sealer	ABGene	AB-0384
• Adhesive seals	384-Well Microplate Aluminum Sealing Tape	Corning	6569
	Adhesive PCR foil seal	ABGene	AB-0626
	Silverseal	Greiner	676 090
GeneMapper® Software v3.7[b]		See your Applied Biosystems representative for information.	
Data Collection Software v2.0 or higher		See your Applied Biosystems representative for information.	

a. IMPORTANT! Applied Biosystems has found that certain plate covers negatively affect the performance of the SNPlex System assay. If you use covers other than the recommended plate covers, test them using the SNPlex™ System Control Set (see Appendix A).

b. Modules for GeneMapper and Data Collection Software are available at **http://www.appliedbiosystems.com/support/software**.

Reagents

Table 1-4 Required reagents

Item	Vendor	Part Number
Hi-Di™ Formamide	Applied Biosystems	4311320
Sterile 1X TE buffer (10 mM Tris-base, pH 8.0, and 1 mM Na_2EDTA)	Fluka	93283
0.1 N NaOH	Major Laboratory Supplier (MLS)	—
Nuclease-free water	Promega	P119C

Other Required Materials

Table 1-5 Other required materials

Item	Vendor	Part Number
Hybridization oven, capable of maintaining a constant temperature of 37 °C ±1 °C	MLS	—
Centrifuge (equipped to accomodate reaction plates)	MLS	—
Vortex	MLS	—
96-well aluminum block	MLS	—
384-well aluminum block	MLS	—
Multichannel pipettor, 250-µL	MLS	—
Pipetting reservoirs, 25-mL	MLS	—
Pipetting reservoirs, 100-mL	MLS	—
Rotary shaker	MLS	—
Standard heat block	MLS	—
Sterile, wide-bore pipette tips	VWR	46620-642

Optional Materials

Table 1-6 Optional reagents

Function	Item	Vendor	Part Number
DNA Purification (choose one)	Qiagen® Flexigene Kit	Qiagen	51206
	Gentra® Puregene Kit	Gentra	D-5000
DNA Quantitation (choose one)	• TaqMan® RNase P DNA Quantification Kit *or* • TaqMan® RNase P Detection Reagents Kit **Note:** The RNase P kits require one of the TaqMan® Universal PCR Master Mixes listed below.	Applied Biosystems	4343782 *or* 4316831
	• TaqMan® Universal PCR Master Mix *or* • TaqMan® Universal PCR Master Mix without UNG	Applied Biosystems	4304437 *or* 4324018
	PicoGreen® dsDNA Quantitation Kit	Molecular Probes	P-7589
	PicoGreen® dsDNA Quantitation Kit, special packaging	Molecular Probes	P-11496
	PicoGreen® dsDNA Quantitation reagent	Molecular Probes	P-7581
	PicoGreen® dsDNA Quantitation reagent, special packaging	Molecular Probes	P-11495

Chemistry Overview

Component Kits

Several kits and reagents are required for the SNPlex System Assay, as specified in "Kits and Reagents" on page 1-8. These kits and reagents include the associated enzymes, master mixes, and other components required to perform each step in the SNPlex System Assay.

Workflow

Figure 1-3 summarizes the processes required to perform the SNPlex™ System Assay. For simplicity, the figure shows the assay for a single SNP allele.

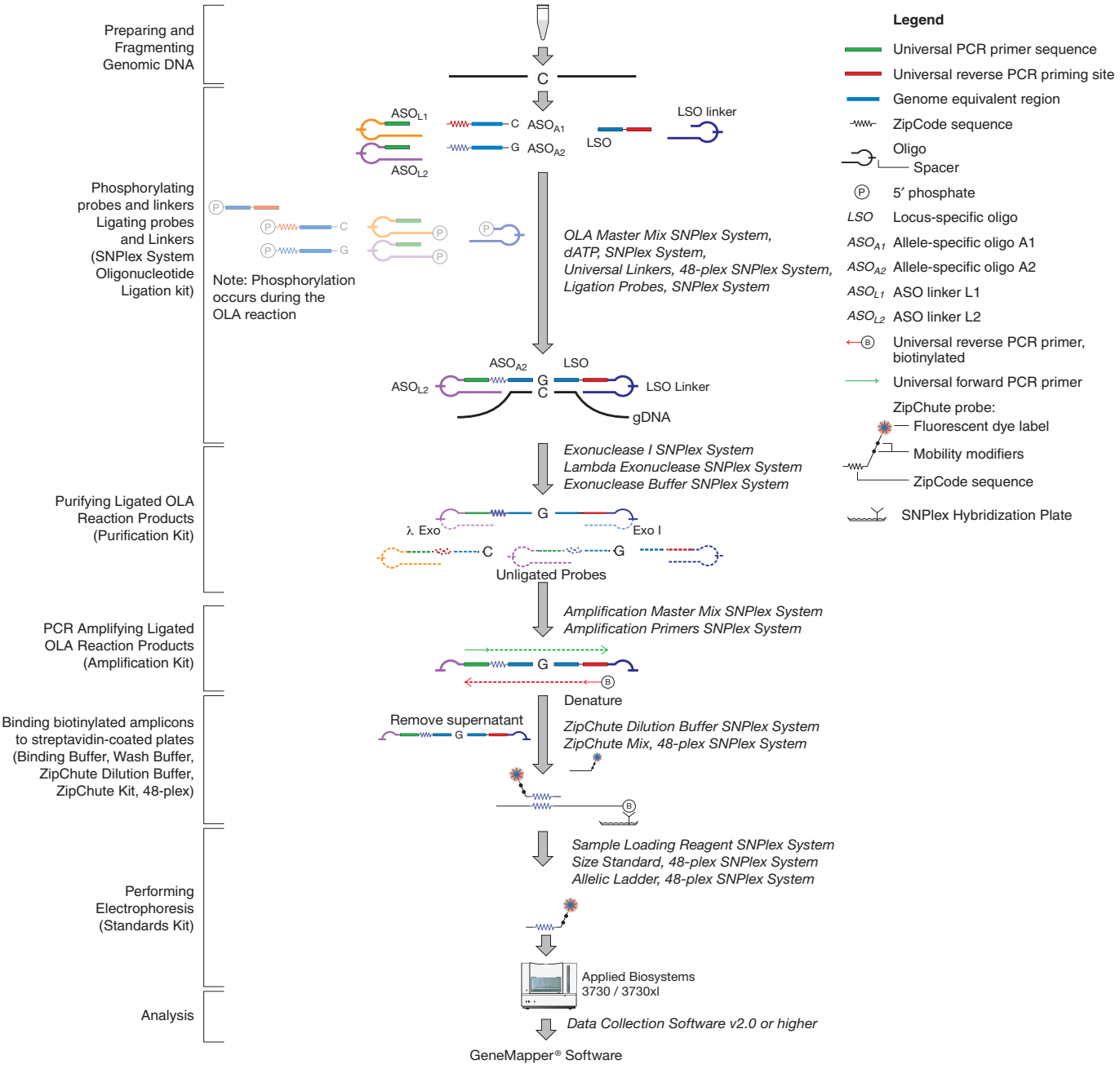


Figure 1-3 Summary of the SNPlex System Assay process

Preparing and Fragmenting Genomic DNA

To prepare the genomic DNA (gDNA) samples, you need to:

- Purify the DNA sample.
- Determine the concentration of the DNA.
- Fragment and dilute DNA.
- Optionally, dry-down the DNA.

IMPORTANT! Perform procedures related to purifying, fragmenting, and determining concentration of DNA in an amplicon-free environment (see "Laboratory Design: Preventing Amplicon Contamination" on page 1-25).

About Purification

Because most laboratories have their own methods for purifying samples, Applied Biosystems offers only guidelines rather than a specific procedure for purifying genomic DNA (gDNA). gDNA purified for SNP analysis should have:

- A concentration of DNA between 50 to 200 ng/µL
- A length of ≥12 kb before boiling
- A low level of protein contamination
- Low levels of PCR-inhibiting substances such as guanidine hydrochloride, heme, isopropanol, and ethanol

Kits for Purifying Genomic DNA

Applied Biosystems suggests the following kits for purifying genomic DNA from blood for the SNPlex System assay:

- Qiagen Flexigene Kit (PN 51206)
- Gentra Puregene Kit (PN D-5000)

Whole Genome Amplification

Consider using whole genome amplification (WGA) if you encounter problems arising from insufficient quantities of gDNA. When using WGA, consider the

- **Quality of gDNA** – Use only high-quality gDNA for WGA.
- **Input quantity of gDNA** – Use at least 10 ng of gDNA to avoid allelic imbalance and under-representation.
  - If the gDNA is degraded (low quality), consider using higher input concentrations.
  - Consider using higher concentrations of amplified DNA during the ligation step.

About Fragmentation

To produce the most consistent results for SNPlex System genotyping, fragment the DNA by boiling.

Before fragmenting DNA, verify that all samples have comparable quality. Fragmenting degraded samples leads to over-fragmented DNA, which in turn leads to poorly-clustered genotypes.

To check the quality of the DNA, run an aliquot of each sample on a 0.8% agarose gel (see Figure 1-4 on page 1-16). High-quality DNA appears as a solid, high-molecular-weight band. Degraded DNA appears as a smear. If DNA is already degraded before fragmentation, omit the heat-fragmentation step.

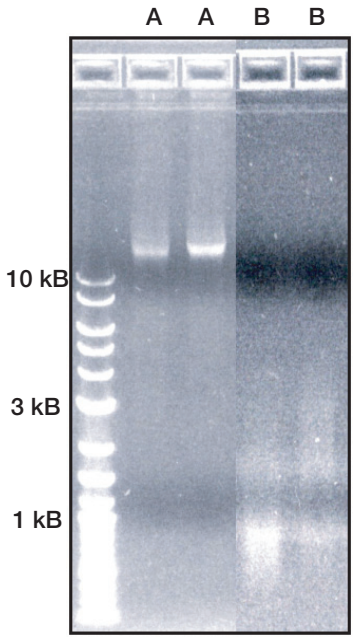


Figure 1-4 High molecular weight gDNA, before heat fragmentation (A), and after 10 minutes fragmentation in 1× TE, pH 8.0 at 99 °C (B)

About Quantification

Applied Biosystems recommends quantifying the concentration of human gDNA using the TaqMan® RNase P DNA Quantification Kit, which provides both quantitative and qualitative assessment of DNA. Fluorescence- or absorbance-based assays, such as the PicoGreen® assay, are more sensitive to the DNA preparation process and can result in "falsely" high calculations of gDNA concentration. In such cases, Applied Biosystems recommends using double the gDNA concentration during the ligation step (as recommended when quantifying the gDNA with the TaqMan® RNase P Quantification Assay).

IMPORTANT! Take care when quantifying gDNA. To obtain tight genotype clusters, the amounts of the different gDNA samples used for the ligation step must be relatively equal. When possible, run an aliquot of the quantified gDNA samples on a 0.8% agarose gel to verify equal gDNA concentration.

TaqMan RNase P Kits

Applied Biosystems recommends quantifying the concentration of human gDNA using the TaqMan® RNase P DNA Quantification Kit or TaqMan® RNase P Detection Reagents Kit. Both kits require TaqMan® Universal PCR Master Mix. Using either kit for quantifying DNA is optional. The assay provides a functional evaluation of the quality of DNA.

IMPORTANT! If using RNase P kits to quantify DNA, quantify the DNA before fragmenting it.

There are two copies of the RNase P gene per human cell. After RNase P reagents bind to the RNAse P gene, the gene is amplified by PCR. During amplification, the RNase P gene probe is cleaved, generating a reporter signal. By referencing a standard curve of RNase P gene concentration in human gDNA, you can interpolate your starting concentration of gDNA.

Item	Applied Biosystems Part Number
TaqMan® RNase P DNA Quantification Kit • 20× RNase P primer and probe mix • Human DNA standard	4343782
TaqMan® RNase P Detection Reagents Kit • 20× RNase P primer and probe mix sufficient to run 200 25-µL reactions	4316831

For a description of how to run the protocol on the ABI PRISM® 7000, 7700, or 7900HT Sequence Detection Systems, refer to *Human DNA Sample Quantification Protocol Using the RNase P Kit* (PN 4342582).

PicoGreen Kits

IMPORTANT! If you use PicoGreen Kits to quantify DNA, quantify the DNA before fragmenting it.

The PicoGreen® dsDNA Quantitation Reagents and Kits from Molecular Probes are also available for double-stranded DNA quantitation.

Note that fluorescence-based assays are more sensitive to the quality of the gDNA preparation, and consequently poor DNA quality can lead to falsely high values for gDNA concentration. To achieve tight genotype clusters with the SNPlex System assay, consider increasing the amount of input gDNA.

Item	Molecular Probes Part Number
PicoGreen® dsDNA Quantitation Kit	P-7589
PicoGreen® dsDNA Quantitation Kit, special packaging	P-11496
PicoGreen® dsDNA Quantitation reagent	P-7581
PicoGreen® dsDNA Quantitation reagent, special packaging	P-11495

Phosphorylating and Ligating Probes to gDNA (OLA)

Protocol Summary

The SNPlex™ System Oligonucleotide Ligation Kit allows you to perform several reactions of the SNPlex System assay workflow simultaneously (see "Phosphorylating and Ligating Probes to gDNA (OLA)" on page 3-9).

Step	Description
Prepare the pooled SNPlex System ligation probe pool.	Thaw a multiplexed set of SNPlex System ligation probes specific for up to 48 SNPs. Three probes are used to interrogate each SNP. • Two of the probes are allele-specific oligos (ASOs). These are designed specifically for the polymorphism by having the discriminating nucleotide on the 3′ end. Each ASO probe sequence also contains a unique ZipCode™ sequence for ZipChute™ probe binding. • The third probe is a locus-specific oligo (LSO). Its sequence is common to both alleles of a given locus and anneals adjacent to the SNP site on its target DNA. The LSO probe contains a partial binding site for a universal reverse primer. In a 48-plex reaction, there are 96 ASOs and 48 LSOs, for a total of 144 SNP-specific oligos.
Prepare the universal linkers.	Thaw the universal linkers. (The same pool of linkers is compatible with all ligation probe pools.) Three linkers are used for each SNP: • Two of the linkers anneal to the two ASOs. These linkers contain – A PCR primer sequence corresponding to the universal forward primer (UA sequence) – A partial cZipCode sequence • The third linker anneals to the LSO and has a universal sequence that is compatible with all LSOs. The sequence includes a partial binding site for a universal reverse primer. In a 48-plex reaction, there are 96 ASO linkers and a single LSO linker (which anneals to all LSOs, regardless of sequence), for a total of 97 linkers.
Prepare the OLA reaction mix.	Thaw the OLA master mix and dATP and combine them with the SNPlex System ligation probes and universal linkers.

Assemble the OLA reaction	Dispense the OLA reaction mix (containing OLA Master Mix, dATP, ligation probes, and universal linkers) into the wells of a reaction plate that contains either dried gDNA or wet gDNA. Reserve the appropriate number of wells, as indicated in the following table.
Thermal-cycle the OLA reactions	Under temperature-controlled conditions, enzyme phosphorylates the ASO and LSO linkers and ligation probes, the linkers anneal with their respective ligation probes, and one or both of the 96 ASO probes and one of the 48 LSO probes per locus bind to the gDNA sample. Ligase promotes the ligation of linkers with their respective ligation probes and the ligation of ASO and LSO probes. UNG enzyme present in the OLA master mix prevents the re-amplification of dU-containing accidental carryover PCR products.

Instrument	Plate Type	NTC	Ctrl DNA	Allelic Ladder	Total # Controls	Total # Samples
3730*xl* (96-capillary)	96-Well	1	1	2	4	92
	384-Well	4	4	8	16	92 × 4
3730 (48-capillary)	96-Well	2	2	4	8	88
	384-Well	8	8	16	32	88 × 4

The OLA Procedure

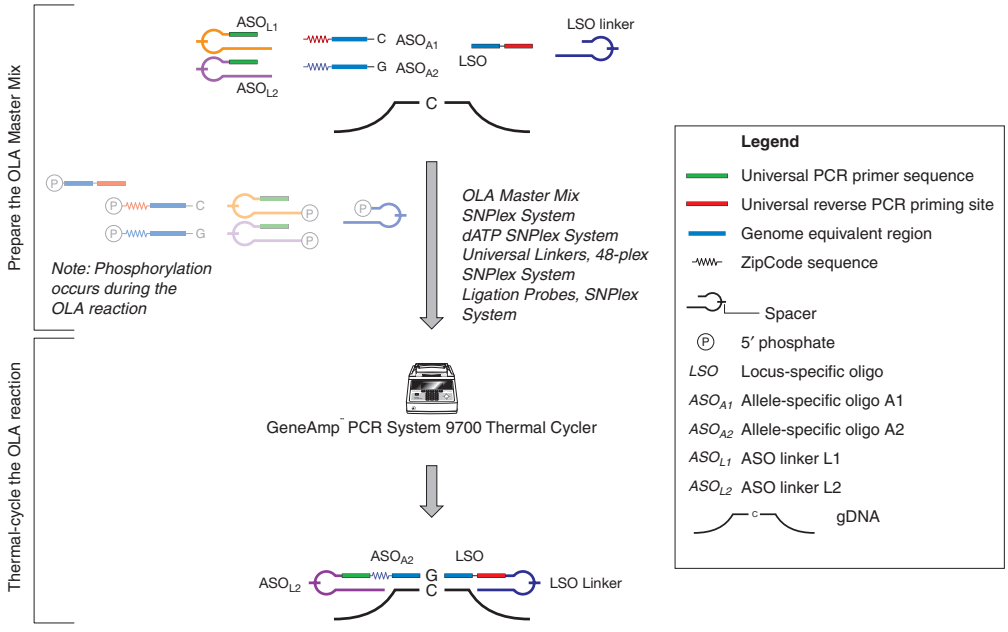


Figure 1-5 OLA procedure

Purifying Ligated OLA Reaction Products

Protocol Summary

The SNPlex™ System Purification Kit uses two exonucleases to digest portions of the ligated OLA reaction products, unligated and partially ligated oligonucleotides, and gDNA (see "Purifying Ligated OLA Reaction Products" on page 3-14).

Step	Description
Perform the exonuclease digestion	Prepare a 2× Exonuclease mix. Add the 2× Exonuclease mix to the OLA reaction to digest the appropriate DNA sequences, including • Unligated oligonucleotides • Ligated products not protected by linker spacers • 5′ portion of ASO linker of correct OLA product, to permit primer annealing • 3′ portion of LSO linker of correct OLA product, to permit primer annealing • gDNA Spacers protect a complete ligation product from exonuclease digestion.
	Dilute purified ligation products with nuclease-free water.

The Purification Procedure

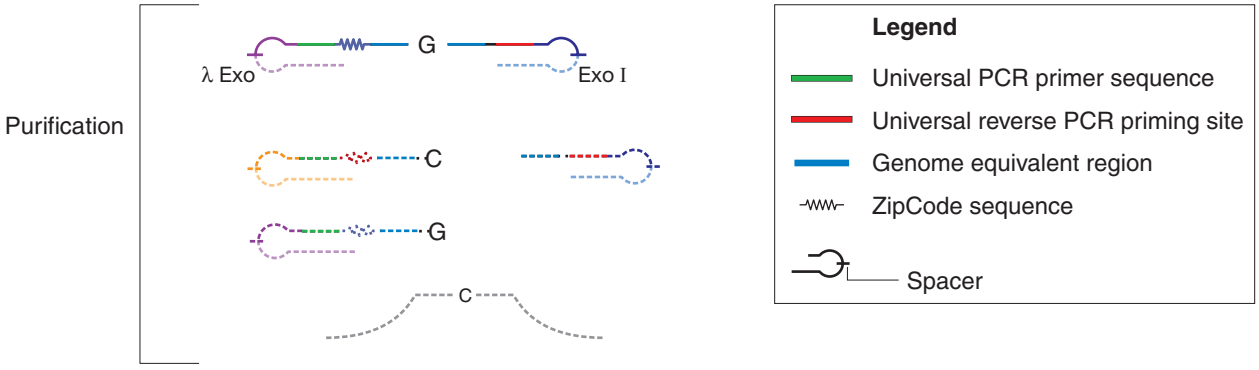


Figure 1-6 Purification procedure

PCR Amplifying Ligated OLA Reaction Products

Protocol Summary

The SNPlex™ System Amplification Kit allows you to amplify the purified and diluted OLA reaction products (see "Performing PCR" on page 3-16).

Step	Description
Prepare the PCR master mix	The Amplification Master Mix, SNPlex System contains buffer and enzyme. The Amplification Primers, SNPlex System contains two universal primers: • The universal forward primer is unlabeled. • The universal reverse primer is biotinylated. Combine the Amplification Master Mix with the Amplification Primers to form the PCR master mix.
Assemble the PCR reaction	The diluted, exonucelase digested OLA reaction products are amplified when the universal primers bind and are extended in the presence of enzyme and adequate cycling conditions. The resulting product is a double-stranded amplicon with one biotinylated strand.
Thermal cycle	

The PCR Procedure

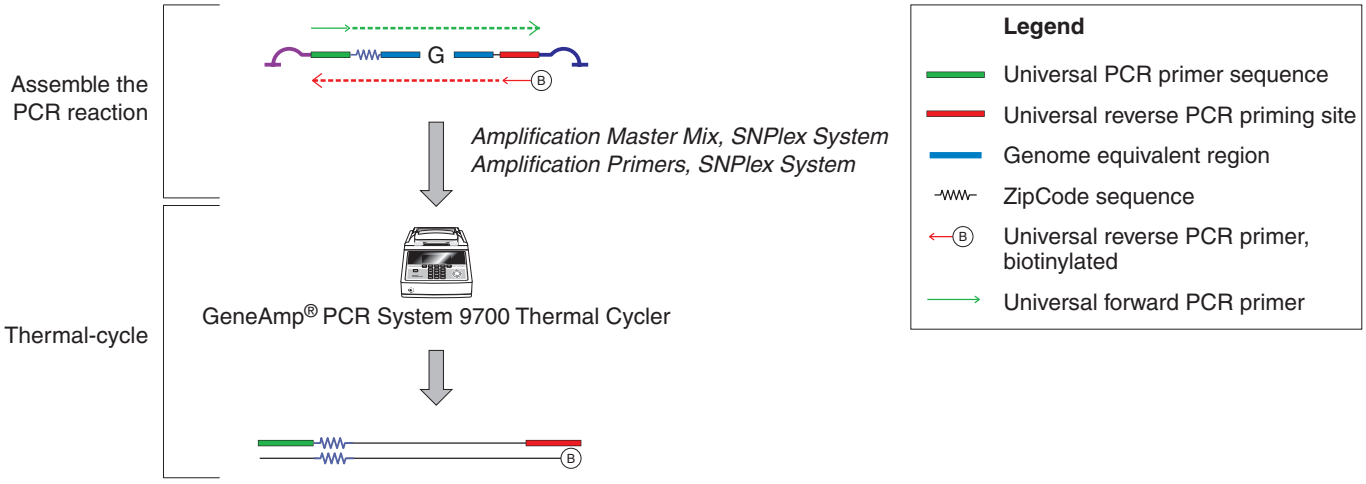


Figure 1-7 PCR amplification procedure

Hybridizing PCR Products to ZipChute Probes and Performing Electrophoresis

Protocol Summary

The hybridization reagents (binding buffer, wash buffer, ZipChute dilution buffer, ZipChute kit) and the SNPlex™ System Standards Kit use fluorescently-labeled ZipChute™ probes and size standards to analyze the results of the assay (see "Hybridizing PCR Products to ZipChute Probes" on page 3-18).

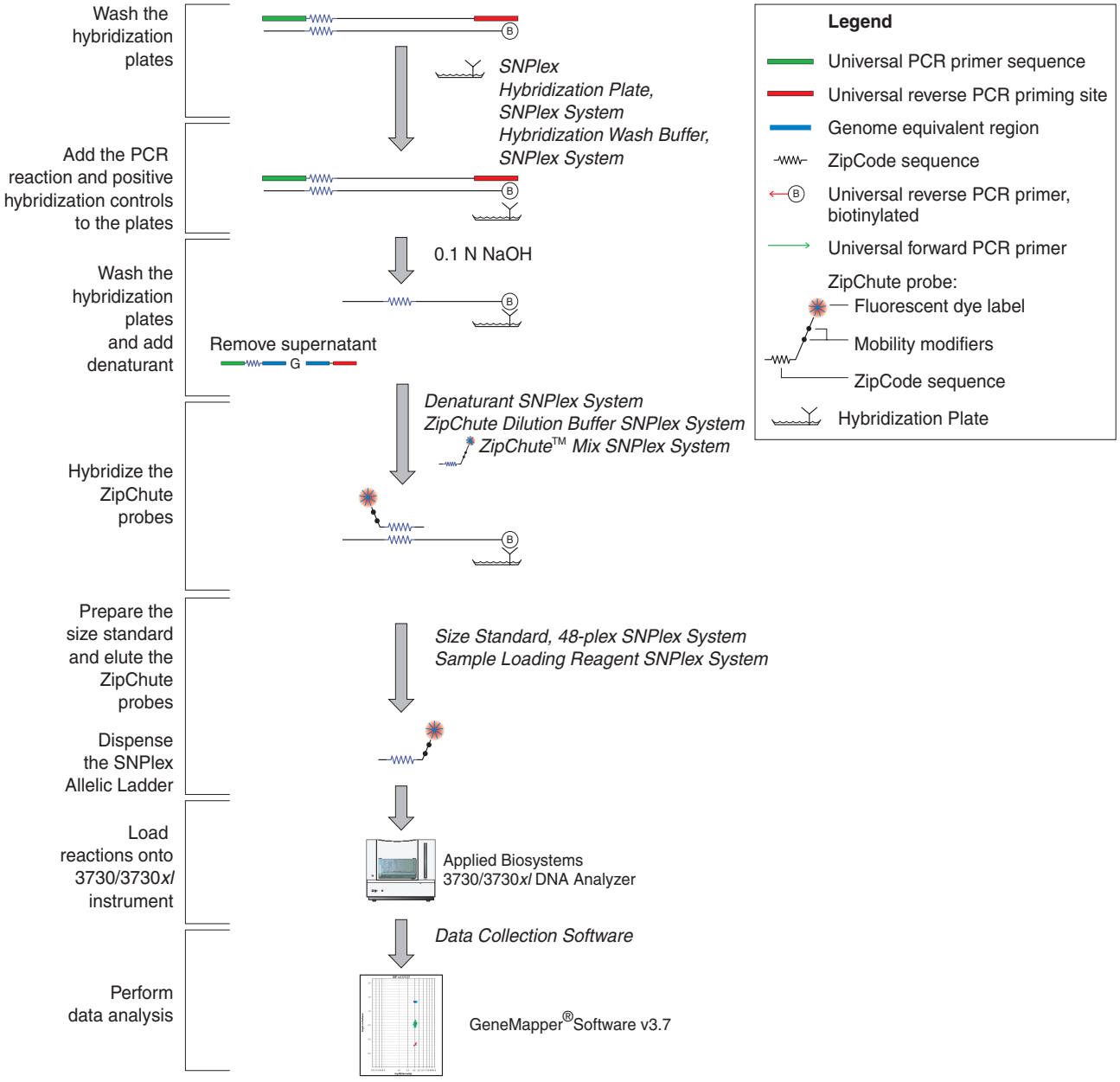
Table 1-7 SNPlex protocol summary

Step	Description
Prepare the hybridization plates, then bind the PCR products to the hybridization plates.	Wash the streptavidin-coated plates and combine the Binding Buffer SNPlex System with the Positive Hybridization Control SNPlex System. Add binding buffer containing positive hybridization control to the hybridization plate, then transfer PCR reactions to the hybridization plate.
Remove unbound material.	Wash the wells to remove unbound material. Add denaturant to separate the strands. The double-stranded amplicon becomes single stranded. The biotinylated strand remains bound to the streptavidin while the unbound strand is washed away.
Hybridize the ZipChute probes to the amplicon.	Prepare the hybridization master mix, then add fluorescently labeled ZipChute probes, which bind specifically to the single-stranded cZipCode™ sequence of the bound, biotinylated PCR strand.
Prepare the sample loading mix.	Combine Size Standard, 48-plex and Sample Loading Reagent to form the sample loading mix. To establish a sizing calibration curve that is used to identify ZipChute probes, each well contains a fluorescently labeled Size Standard, 48-plex. Eleven size-standard (orange) peaks appear in each lane of the electropherogram.
Elute the ZipChute probes.	Add the Sample Loading Mix to the wells and incubate the plate at 37 °C to release the ZipChute probes from the biotinylated strand.

Table 1-7 SNPlex protocol summary *(continued)*

<table>
<tr><td>Dispense the Allelic Ladder, 48-plex</td><td>The allelic ladder is labeled with FAM™ and dR6G dyes. Dispense the ladder into the appropriate number of wells of the sample loading plate, as specified in the following table:</td></tr>
<tr><td colspan="2">
<table>
<tr><th>Instrument</th><th>Protocol</th><th># Allelic Ladder Wells</th></tr>
<tr><td rowspan="2">3730 (48-capillary)</td><td>96-well</td><td>4</td></tr>
<tr><td>384-well</td><td>16</td></tr>
<tr><td rowspan="2">3730xl (96-capillary)</td><td>96-well</td><td>2</td></tr>
<tr><td>384-well</td><td>8</td></tr>
</table>
</td></tr>
<tr><td>Load reactions onto the 3730/3730xl analyzer.</td><td>Load plates onto the 3730/3730xl analyzer to generate sample files. Data analysis is conducted using GeneMapper® Analysis Software v3.7.</td></tr>
</table>

Hybridization and Loading



Laboratory Design: Preventing Amplicon Contamination

Product Design

Preventing amplicon contamination from previous PCR runs is especially important in protocols that use universal primers for all amplifications.

To help prevent amplicon contamination, the SNPlex System kits are designed to be used in a linear workflow: dedicated components used in each step of the assay are not reintroduced in subsequent steps of the workflow.

In addition, the OLA master mix contains uracil-N-glycosylase (UNG). UNG acts on single- and double-stranded dU-containing DNA to cause the release of uracil, creating an alkali-sensitive apyrimidic site in the DNA. UNG has no activity on RNA or dT-containing DNA.

Two Laboratory Areas

In order to minimize the risk of downstream PCR products contaminating upstream OLA reactions, SNPlex System experiments should be conducted in two separate laboratories: OLA and PCR.

OLA Laboratory

In this laboratory, you perform OLA, purify OLA products, and assemble the PCR reactions. You may prepare gDNA samples in the OLA laboratory, or in a separate amplicon-free area.

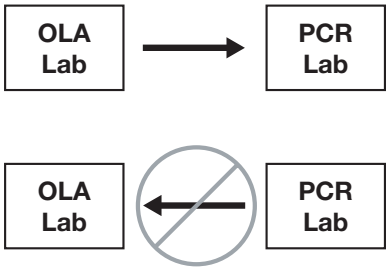
Observe the following precautions:

- Limit access to the OLA lab. As much as possible, a person who enters the PCR lab should not enter the OLA lab for the rest of the day.
- Wear safety goggles, lab coats, gloves, and hair covers that are dedicated to the OLA lab.
- Use a thermal cycler in the OLA lab that has been designated for OLA only.
- Store the following kits and associated materials (for example, microtiter plates and pipette tips) in the OLA lab:
  - SNPlex System Oligonucleotide Ligation Kit (48-plex)
  - SNPlex System Purification Kit (48-plex)
  - SNPlex System Amplification Kit (48-plex)
  - SNPlex System Assay Control Kit
  - SNPlex System Ligation Probes
  - SNPlex System Control Pool, 48-plex
  - SNPlex System Dried gDNA Plates Kit
- Place a sticky mat at the entrance of the OLA lab.

PCR Laboratory

In this laboratory, you thermal cycle the PCR reactions, then proceed with the remaining steps in the SNPlex System assay. You may perform the run on the 3730/3730*xl* analyzers in this laboratory, or in a separate data collection area.

IMPORTANT! Never move equipment, containers, or other items from the PCR Laboratory or data collection area into the OLA laboratory.



Observe the following precautions:

- Wear safety goggles, lab coats, gloves, and hair covers that are dedicated to the PCR lab.
- Use a thermal cycler in the PCR lab that has been designated for PCR amplification only.
- Store the following kits and associated materials (for example, microtiter plates and pipette tips) in the PCR lab:
  - Hybridization Binding Buffer SNPlex System
  - Hybridization Wash Buffer (10×) SNPlex System
  - ZipChute Dilution Buffer SNPlex System
  - SNPlex System ZipChute Kit, 48-plex
  - SNPlex System Standards Kit, 48-plex
  - SNPlex System Hybridization Plates (384-well or 96-well)
  - SNPlex System Matrix Standard DS-40, Dye Set S*
  - SNPlex System Array Conditioning Kit[1]

Additional Precautions

- Use filter-tips for all pipetting steps.
- Routinely decontaminate robotic equipment. Refer to the manufacturer's directions for a procedure.
- Routinely decontaminate thermal cyclers. Refer to the manufacturer's directions for a procedure.
- Routinely decontaminate laboratory work surfaces.

1. If performing the electrophoresis runs in the PCR laboratory.

SNPlex System Assay Workflow

Figure 1-8 summarizes the procedures that you should carry out in each lab.

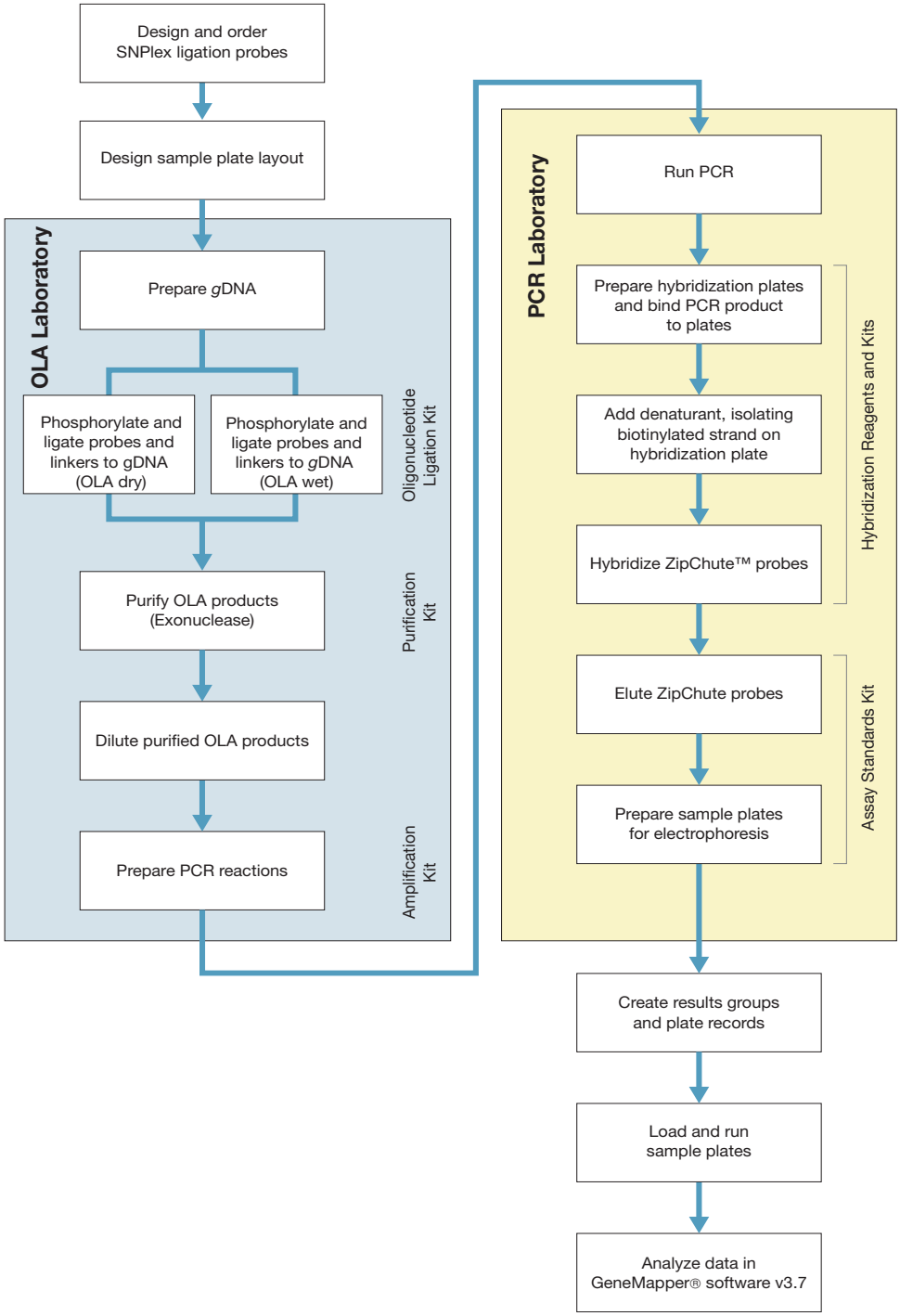


Figure 1-8 Division of procedures between OLA and PCR labs

Setting Up Applied Biosystems 3730/3730*xl* DNA Analyzers for SNPlex System Experiments

2

This chapter covers:

Overview

Setting up the Applied Biosystem 3730/3730*xl* DNA Analyzers for use with the SNPlex™ Genotyping System involves:

- Importing SNPlex™ System modules into the Data Collection software

  Note: Systems upgraded to Data Collection v3.0 have the SNPlex System instrument files pre-installed:

  – PrebatchModule.txt
  – Module HTSNP36_POP7_V3
  – Dye Set S
- Preconditioning the capillary array
- Performing spatial and spectral calibrations
- Validating instrument performance

All files referred to in this chapter are supplied on the *SNPlex™ System 48-plex Support Files CD* (PN 4352129). You can also download the files from the Applied Biosystems Web site at **http://www.appliedbiosystems.com/support/software**.

Supported Configuration

The SNPlex Genotyping System is optimized for use with:

- A 3730/3730*xl* analyzer
- Data Collection software v2.0 or higher
- POP-7™ Performance Optimized Polymer
- A 36-cm capillary array
- GeneMapper® Software v3.7 or higher

Required Materials

Description	Vendor	Part Number
POP-7™ Performance Optimized Polymer	Applied Biosystems	4335615
SNPlex™ System Array Conditioning Kit	Applied Biosystems	4352018
DS-40 Spectral Calibration Standard Kit (Dye Set S)	Applied Biosystems	4349365
10X Running Buffer with EDTA	Applied Biosystems	4335613
36-cm 48-capillary array (3730 analyzer)	Applied Biosystems	4331247
36-cm 96-capillary array (3730*xl* analyzer)	Applied Biosystems	4331244
Hi-Di™ Formamide	Applied Biosystems	4311320
SNPlex™ System Assay Standards Kit	Applied Biosystems	4349351
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems	N8010560

Importing SNPlex System Files into the Data Collection Software

Before the Data Collection software can process SNPlex System data, you must load three files from the *SNPlex System 48-plex Support Files CD* (or download them from the Applied Biosystems Web site):

- PrebatchModule.txt
- HTSNP36_POP7_V2
- S.zip

Note: If you are using Data Collection v3.0, skip to "Creating an Instrument Protocol for SNPlex System Experiments" on page 2-4.

Replacing the PrebatchModule.txt File

To replace PrebatchModule.txt:

1.	On the computer running the Data Collection software, navigate to E:\AppliedBiosystems\UDC\DataCollection\SupportFiles\ga3730\ServiceModules.
2.	Rename the existing PrebatchModule.txt file. For example, OriginalPrebatchModule.txt.
3.	Copy the PrebatchModule.txt file from the Data Collection Files folder on the SNPlex System support CD.
4.	Verify that the first line of the file reads //SNPlex v2.0 prebatch.
5.	Paste the file into the ServiceModules folder.

Importing the HTSNP36_POP7_V2 Module

To import the HTSNP36_POP7_V2 module:

1.	Determine if the module is installed on your computer. a. Start the Data Collection software. b. Open the Module Manager window. c. Check the list of modules.
2.	If the module is not installed, copy the the HTSNP36_POP7_V2.xml file from the Data Collection Files folder in the SNPlex System support CD.
3.	Paste the file into the following directory: E:\AppliedBiosystems\UDC\DataCollection\SupportFiles\ga3730\RunModules
4.	In E:\AppliedBiosystems\UDC\DataCollection\bin, double-click the **Import3730RunModules.exe** file.

To import the HTSNP36_POP7_V2 module: ***(continued)***

5.	Verify that the module has been installed by opening the Module Manager window and observing that HTSNP36_POP7_V2_1 appears in the module list.
6.	In the Module Manager, click **Edit,** then verify that the module has a 45-second prerun at 15 kV.

Installing Dye Set S

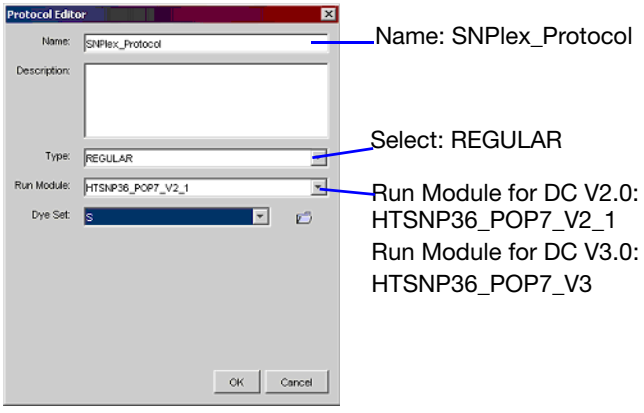
To install Dye Set S:

1.	Start the Data Collection software.
2.	Open the Protocol Manager.
3.	Start a new spectral calibration using Dye Set S. • If the protocol is not available, install it as explained in step 4. • If the protocol is available, exit this procedure.
4.	In the Protocol Manager, click **New**.
5.	Click the folder icon, then navigate to the S.zip file in the Data Collection Files folder in the SNPlex System support CD.
6.	Click **Open**. Dye Set S should now be available.

Creating an Instrument Protocol for SNPlex System Experiments

An instrument protocol contains all the setting necessary to run the instrument.

To create an instrument protocol:

1.	In the Tree pane of the Data Collection Software, click **GA Instruments > ga3730** or **ga3730xl > Protocol Manager**.
2.	In the Instrument Protocols section, click **New**. The Protocol Editor opens.
3.	Complete the Protocol Editor as shown in the figure below. 
4.	Click **OK** to save the instrument protocol.

Preconditioning the Capillary Array

Before running SNPlex System chemistry on 3730/3730*xl* analyzers—that is, before performing spectral or spatial calibrations or SNPlex System protocols—you must precondition the capillary array. Runs performed using improperly conditioned arrays have poorly resolved peaks.

To precondition the capillary array:

1.	Using the reagents in the SNPlex System Array Conditioning Kit, prepare a 500× dilution of Array Conditioning Buffer. a. Dispense 100 mL of molecular biology-grade, deionized water into a sterile graduated cylinder. b. Add 200 µL of Array Conditioning Buffer (included in the SNPlex™ System Array Conditioning Kit, PN 4352018). c. Cover and invert several times to mix.
2.	Rinse the plastic array header shipping cover (supplied with the new array) with deionized water.
3.	Pour the diluted Array Conditioning Buffer solution into the cover, then place the array into the solution-filled cover.
4.	Assemble the 20-mL syringe, Luer adaptor, and tubing supplied in the SNPlex™ System Array Conditioning Kit (PN 4352018). Slip the open end of the tubing over the capillary bundle at the detection end of the array.
5.	Using the syringe, pull enough of the diluted Array Conditioning Buffer solution to fill the array. Ensure that the array is filled by looking at the detection cell. Capillaries filled with Array Conditioning Buffer solution appear dark. Additionally, a small volume of solution enters the attached tubing.
6.	Allow the solution to incubate in the array for 30 min. Make sure that the array tips are submerged in the solution.
7.	Remove the syringe assembly, then rinse the array bundle end briefly with deionized water.
8.	Install the array on the 3730/3730*xl* analyzer using the Array Install wizard. You do not need to rinse the array interior before installing it on the instrument. Select the optional extra array fill at the end of the wizard.
9.	Change the buffer (cup and tray), water, and waste reservoirs.

Performing Spatial and Spectral Calibrations

Performing a Spatial Calibration

The 3730/3730*xl* Data Collection Software uses images collected during spatial calibration to establish a relationship between the signal emitted by each capillary and the position where that signal falls and is detected by the CCD camera.

Perform a spatial calibration after you:

- Install a new or used capillary array
- Remove the capillary array from the detection cell block (even to adjust it)
- Move the instrument (even if the instrument was moved on a table with wheels)

To perform a spatial calibration:

1.	In the Data Collection software navigation pane, select the Spatial Run Scheduler.
2.	Select the **SpatialFill_1** module.
3.	Perform the spatial calibration as described in the *Applied Biosystems 3730/3730xl DNA Analyzers Getting Started Guide* (PN 4331468).

Performing a Spectral Calibration

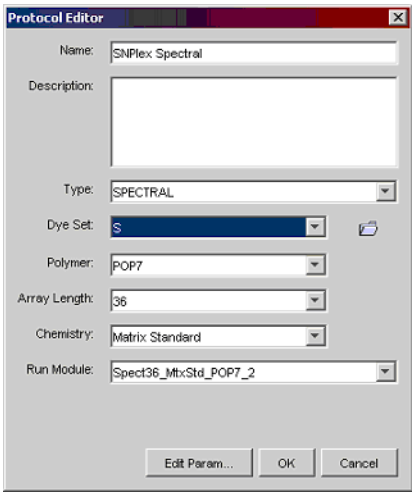
The SNPlex™ Matrix Standard DS-40 (PN 4349365) is used to generate the "multicomponent matrix" required when analyzing 6FAM™, dR6G, BigDye®-TAMRA™, BigDye®-ROX™, and LIZ®-labeled DNA fragments on the Applied Biosystems 3730/3730*xl* DNA Analyzers. The Data Collection Software for these instruments uses the multicomponent matrix to automatically analyze the five differently colored fluorescent dye-labeled samples in a single capillary.

You do not need to run matrix standards with every set of sample injections. However, you do need to run the standards once in order to generate a matrix file that is then applied to samples run under similar conditions. For more information on the use of matrix standards, refer to the instrument User's Manual.

The SNPlex™ System Matrix Standard kit consists of one tube of matrix standard, which is sufficient for a minimum of eight array runs on the 3730*xl* analyzer and 16 runs on the 3730 analyzer. The SNPlex™ System Matrix Standard contains five specific sizes of DNA fragments labeled with a unique fluorescent dye label. This standard is formulated in buffer and is stable for one year when stored at 2 °C to 8 °C. Do not freeze. Avoid exposure to light.

WARNING **CHEMICAL HAZARD**. **Formamide**. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the matrix standard for the 3730 or 3730*xl* analyzers:

1.	Thaw and thoroughly mix the contents of the tube and spin briefly in a microcentrifuge.
2.	Prepare a spectral calibration sample by combining: • 900 µL Hi-Di™ Formamide (PN 4311320) • 100 µL DS-40 Spectral Calibration Standard (PN 4349365)
3.	Heat the mixture at 95 °C for 5 min to denature the DNA fragments, then immediately place on ice. **Note:** Make samples fresh for each run. Samples can be stored refrigerated for up to 8 hours. Discard excess material.
4.	Dispense 5 µL (384-well plates) or 10 µL (96-well plates) of the spectral calibration sample into the appropriate number of wells. • 48 wells for a 3730 analyzer • 96 wells for a 3730*xl* analyzer For instructions on setting up a plate for a 48-capillary array or a 96-capillary array, refer to the *Applied Biosystems 3730 / 3730xl DNA Analyzers User Reference Guide* (PN 4331468).
5.	Centrifuge the plate to ensure that the samples are at the bottom of the wells.
6.	Create a spectral instrument protocol in the Protocol Manager, as shown in the figure below.  For details on setting up a run, refer to the *Applied Biosystems 3730 / 3730xl DNA Analyzers User Reference Guide* (PN 4331468).

To prepare the matrix standard for the 3730 or 3730*xl* analyzers: *(continued)*

7.	Create a plate record for the spectral calibration as explained in the *Applied Biosystems 3730/3730xl DNA Analyzers Getting Started Guide* (PN 4331468). Briefly, a. Select **Plate Manager**. b. Click New, then complete the following fields: – Plate ID: Enter an ID for the plate. – Plate Name: Enter a name for the plate. – Application: Select **Spectral Calibration**. – Plate Type: Select **96-well** or **384-well**, as appropriate. – Plate Seal: Select **Septa** or **Heat Seal**, as appropriate. – Owner Name: Enter a name. – Operator Name: Enter a name. c. Click **OK**. A blank sample sheet appears. d. Complete the following fields: – Sample Name – Instrument Protocol: Select the instrument protocol that you created in step 6 on page 2-4.
8.	Place the plate with the spectral calibration samples into the In Stack.
9.	Click **Run**. For details on how to perform a spectral calibration, refer to the *Applied Biosystems 3730/3730xl DNA Analyzers Getting Started Guide* (PN 4331468).

Validating Instrument Performance

To assess signal intensity and resolution, you must perform a mock run using a diluted solution of the SNPlex™ ZipChute™ Mix and an internal size standard.

Preparing the Test Sample Plate

WARNING **CHEMICAL HAZARD. SNPlex Sample Loading Reagent** causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the test sample plate:

1.	Dilute the ZipChute Mix by combining: • 2 µL ZipChute Mix, 48-plex (from the SNPlex™ System ZipChute™ Kit, 48-plex, PN 4349361) • 448 µL molecular-biology-grade deionized water
2.	Vortex thoroughly.

To prepare the test sample plate: *(continued)*

3.	Prepare the test sample by mixing: • 920 µL SNPlex Sample Loading Reagent (from the SNPlex System Assay Standards Kit, (PN 4349351) • 40 µL of SNPlex Size Standard (from the SNPlex System Assay Standards Kit, PN 4349351) • 40 µL of the diluted ZipChute™ Mix
4.	Vortex thoroughly.
5.	Dispense 10 µL of the spectral calibration sample into the appropriate number of wells of a MicroAmp® Optical 96-Well Reaction Plate. • 48 wells for a 3730 analyzer • 96 wells for a 3730*xl* analyzer
6.	Complete the plate record, selecting the instrument protocol you created for SNPlex System experiments ("Creating an Instrument Protocol for SNPlex System Experiments" on page 2-4).
7.	Start the run.

Evaluating the SNPlex System Run

To evaluate the sample run, review the sample data from each well of the sample plate using the History View of the Data Collection software.

All 11 size-standard peaks (shown in orange) should be approximately the same height and width.

Figure 2-1 shows examples of acceptable and poor resolution.

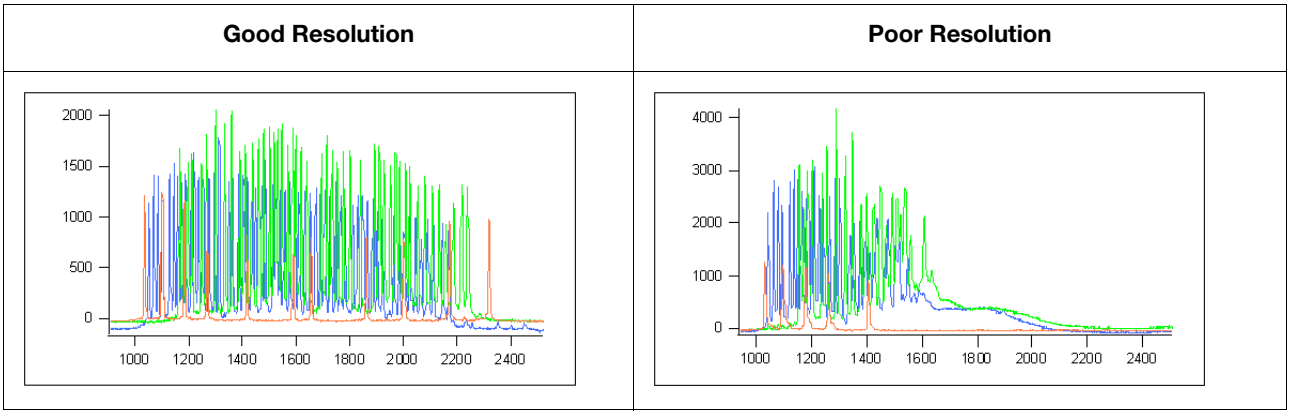


Figure 2-1 Example of acceptable and poor resolution

SNPlex System Protocols 3

This chapter covers:

Overview

Protocols in This Guide

This chapter provides all the protocols necessary to manually perform SNPlex® System experiments using 96- or 384-well plates on the 3730/3730*xl* analyzers. All volumes are for single reactions and need to be scaled-up appropriately.

The *SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide* (PN 4358099) provides modified protocols for automating the SNPlex System assay using robotics.

Figure 3-1 illustrates the workflow for SNPlex System experiments.

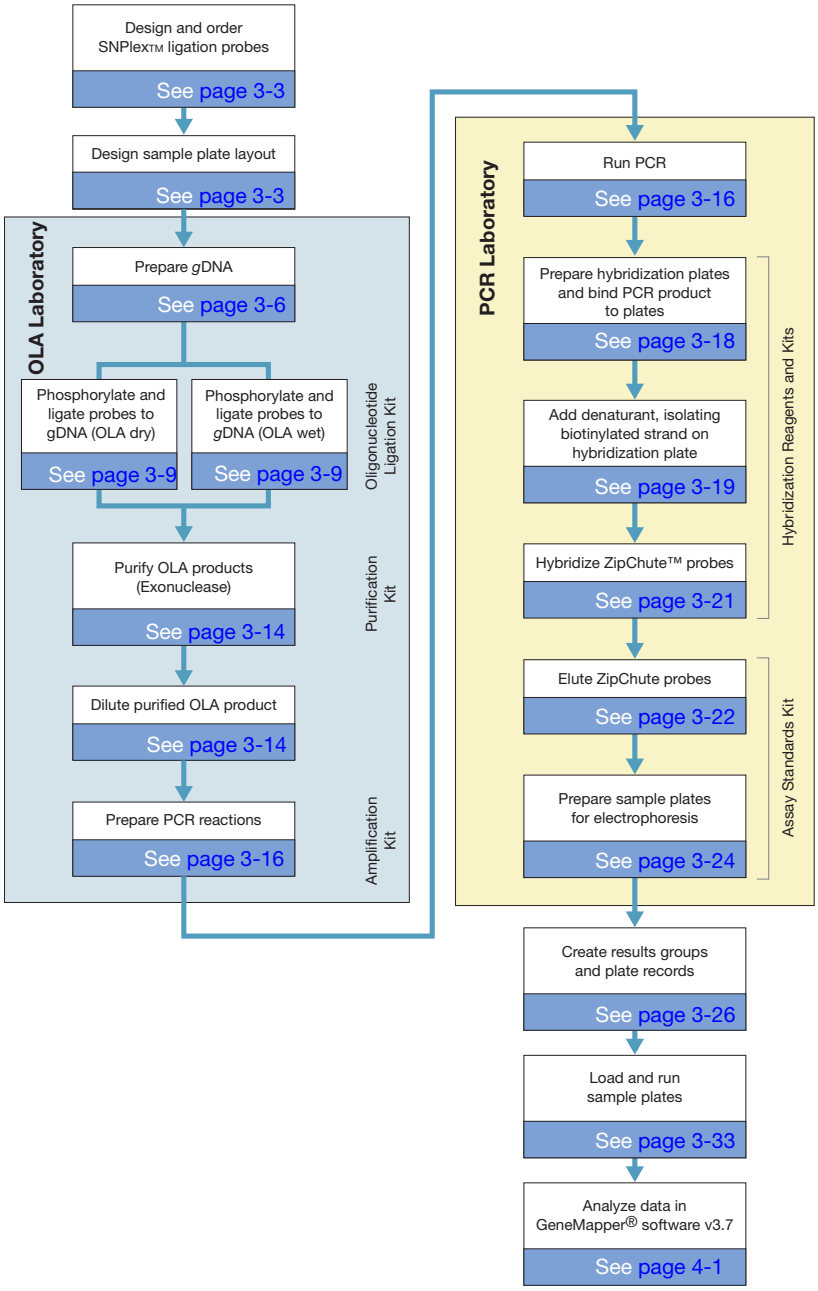


Figure 3-1 SNPlex System experiment workflow

Before You Begin

At this point, you should already have ordered your SNPlex System probe pools. If you have not done so, refer to the *SNPlex™ Genotyping System Assay Ordering Guide* (PN 4357460) for information about designing and ordering SNPlex System probe pools.

Designing the Sample Plate Layout

Purpose

To analyze SNPlex System data, GeneMapper® software requires that each run:

- Includes at least one allelic ladder sample, which allows GeneMapper software to perform sizing bin adjustments on a per-run basis, greatly reducing binning errors.
- Has a unique run folder set up in the Data Collection software. All samples from a run must be saved in a unique run folder.

Coordinating the layout of your sample plates with the structure and naming of Data Collection software run folders allows the software to organize SNPlex System data into folders grouped by probe pool and run.

IMPORTANT! Combining sample plate layout with the proper run folder naming convention (explained in "Setting Up Results Groups" on page 3-26) allows the Data Collection software to organize data into folders grouped by probe pool and instrument run. This organization is the required data structure for GeneMapper software to perform clustering analysis.

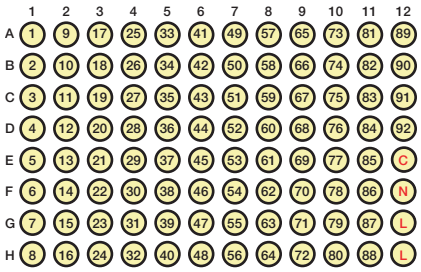
This section describes recommended plate layouts. Refer to "Setting Up Results Groups" on page 3-26 for information on setting up results groups for SNPlex System experiments.

Assumptions

The following illustrations provide examples of sample layouts for 384-well and 96-well plates. The setups assume that there are four probe pools per 384-well plate and one probe pool per 96-well plate. The number of gDNA samples, controls, NTCs, and allelic ladders differs between 96-capillary and 48-capillary instruments.

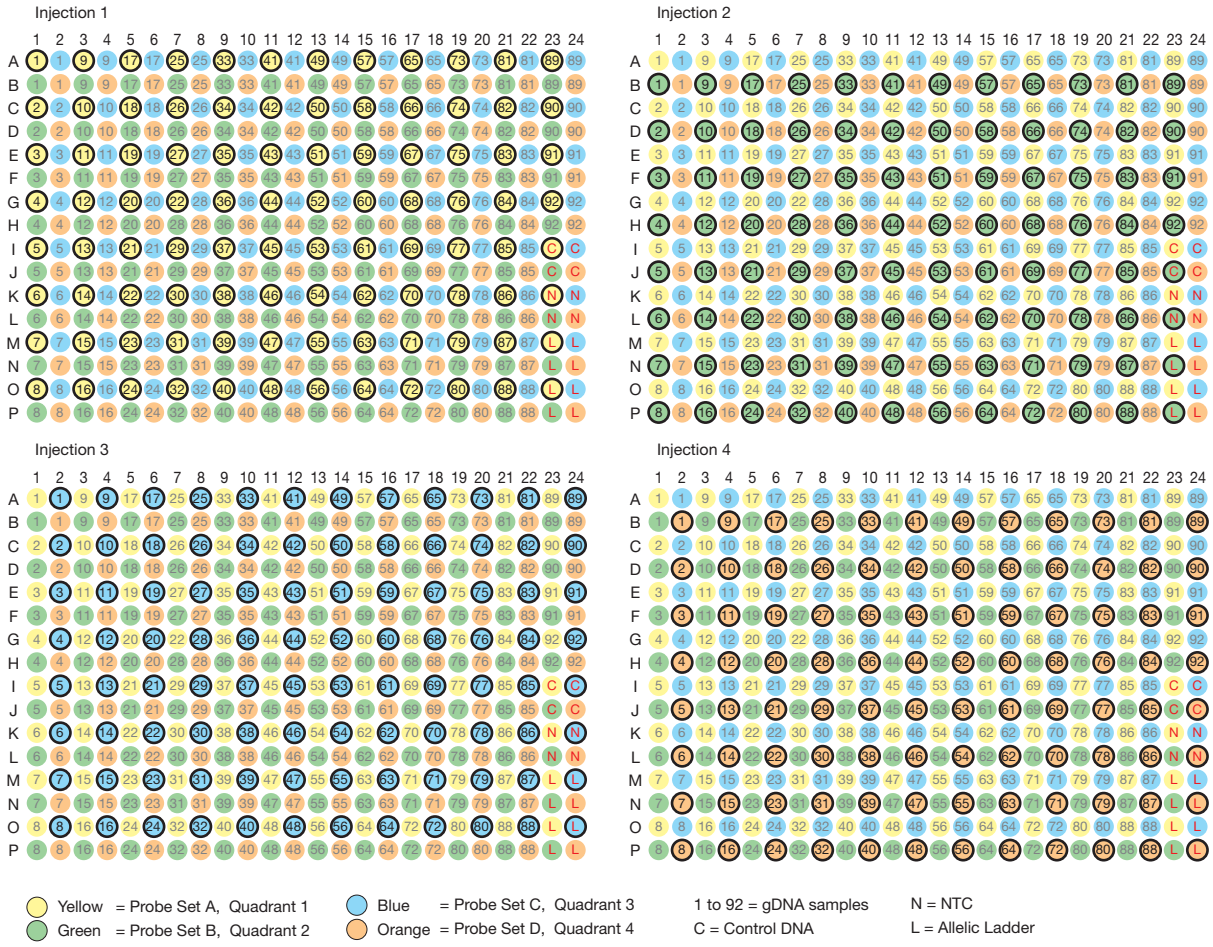
3730*xl* Instrument (96-capillary), 96-wells

An instrument running a 96-capillary array injects once, picking up contents from each of the 96- wells of the plate and performing a single run.



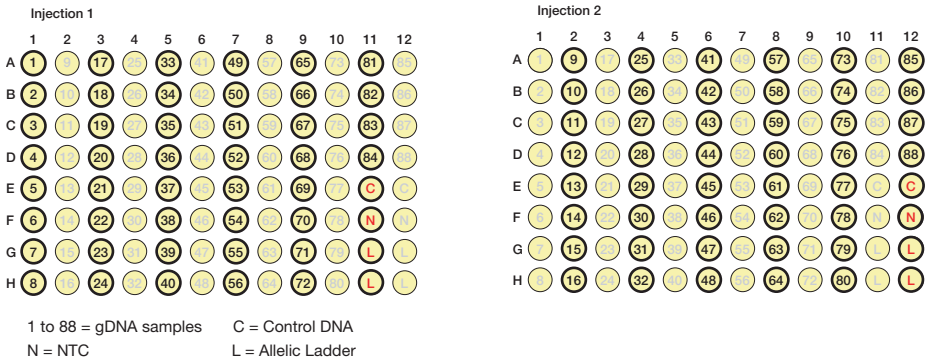
3730xl Instrument (96-capillary), 384-wells

An instrument running a 96-capillary array injects once from each of the four quadrants of a 384-well plate, performing four separate runs.



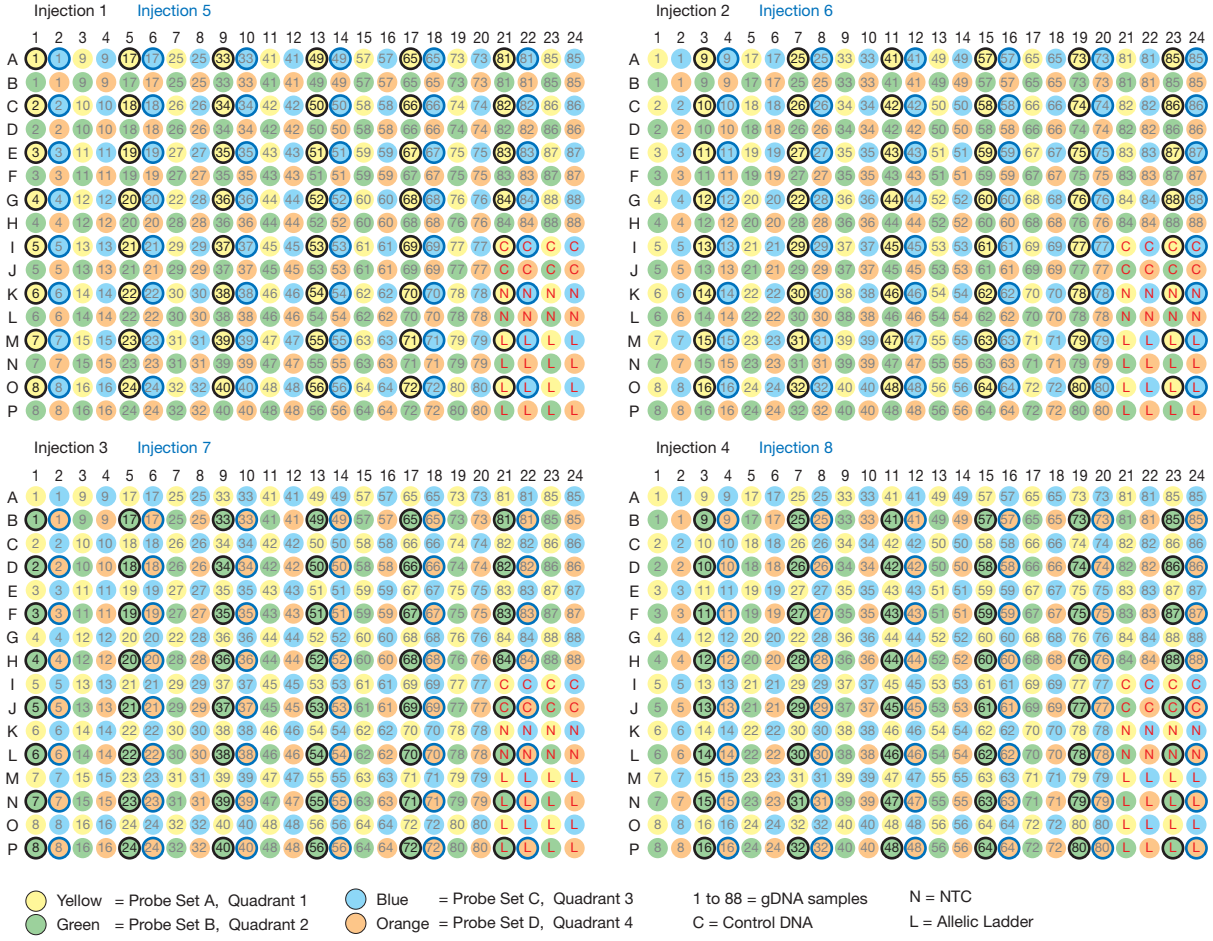
3730 Instrument (48-capillary), 96-wells

An instrument running a 48-capillary array injects twice from a 96-well plate, picking up contents from half of the wells (48 wells per injection) and performing two separate runs.



3730 Instrument (48-capillary), 384-wells

An instrument running a 48-capillary array injects twice from each of the quadrants of a 384-well plate, performing eight separate runs.

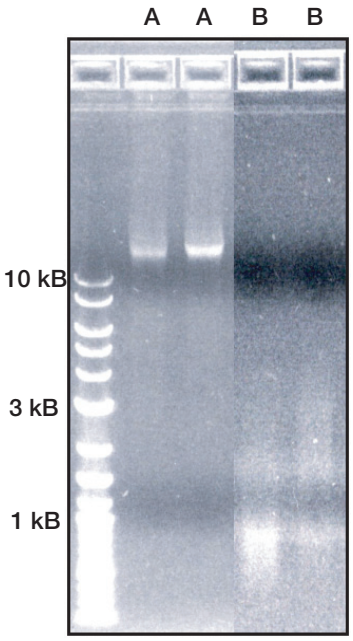


Preparing Genomic DNA

IMPORTANT! Perform all gDNA fragmentation procedures in an amplicon-free environment. Review "Preparing and Fragmenting Genomic DNA" on page 1-15 for important information about this process.

Preparing Purified gDNA

Prepare the purified gDNA for fragmentation:

1.	Purify your gDNA (see page 1-15 for recommended kits).
2.	Determine the concentration of your DNA. Refer to "About Quantification" on page 1-16.
3.	(Optional, but recommended.) Run an aliquot of each quantified DNA sample on a 0.8% agarose gel. • If the sample appears as a solid, high-molecular-weight band, continue with the procedure. • If the sample appears smeared across the lane, omit the heat-fragmentation step.  This figure shows high molecular weight gDNA, before heat fragmentation (A), and after 10 minutes fragmentation in 1✕ TE, pH 8.0 at 99 °C (B).
4.	Using 1✕ TE, pH 8.0[a,b], dilute the purified DNA to a final concentration of between 50 and 200 ng/µL and a final volume between 12.5 and 150 µL. **Note:** The starting concentration of DNA affects the fragment size achieved after boiling. For more dilute DNA samples, you may need to concentrate the DNA or reduce the duration of heating. The duration of heating is determined empirically.

a. 1✕ TE: 10mM TrisHCl, pH 8.0 and 1mM EDTA.

b. Heat fragmentation is equally effective when you dilute purified DNA in nuclease-free water, 0.5✕ TE pH8.0, 2✕ TE pH8.0, 1✕ TE pH 7.5, 1✕ TE pH 7.0, Gentra's PureGene® DNA Hydration Solution, or Qiagen's FlexiGene Hydration Buffer.

Fragmenting the gDNA

To fragment the gDNA:

1. Program the thermal cycler as follows:

Step	Step Type	Temperature (°C)	Time
1	Hold	4	1 min
2	Hold	99	10 min
3	Hold	4	∞

2. Chill a 96-well aluminum block on ice, then place a compatible reaction plate onto it.
3. Dispense up to 150 µL/well of the prepared gDNA onto the chilled reaction plate.
4. Cover the reaction plate.
5. Run the program to boil the gDNA:
   a. Start the thermal cycler.
   b. Pause the program after the thermal cycler block reaches 4 °C.
   c. Insert the chilled reaction plate containing the prepared gDNA.
   d. Resume the program.
6. After the program is complete, remove the reaction plate and place it on the chilled aluminum block.
7. If the same sample was divided into multiple wells, pool the boiled gDNA.
8. Dilute the gDNA to 18.5 ng/µL[a] with 1× TE, pH 8.0.

   Note: If using whole genome amplification (WGA), Applied Biosystems recommends that you dilute the DNA to 37 ng/µL with 1× TE, pH 8.0.

a. The concentration of 18.5 ng/µL is based on quantification using the TaqMan® RNase P Quantification Assay. If you are using fluorescence- or absorbance-based assays, Applied Biosystems recommends using double the gDNA concentration (37 ng/µL).

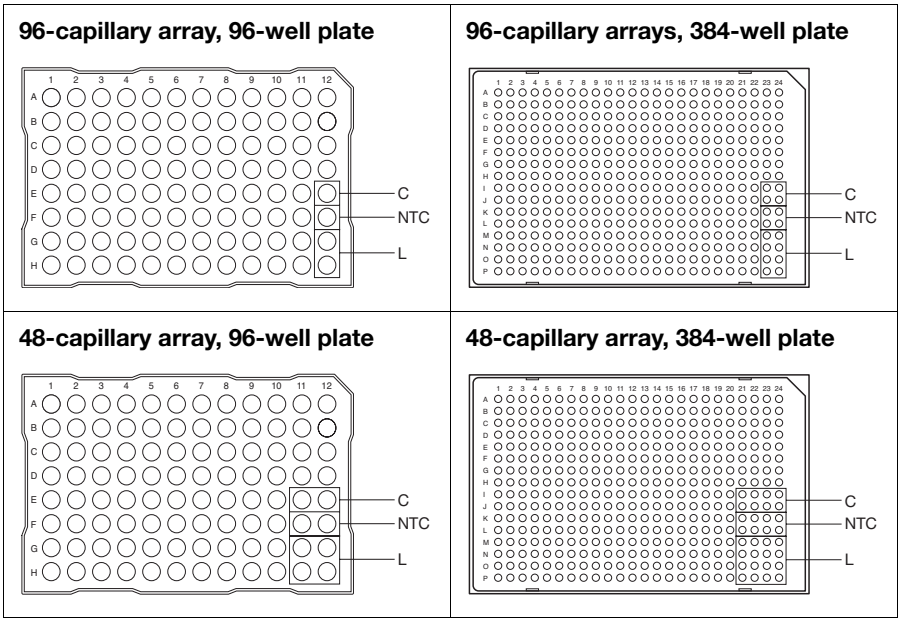
Drying Down gDNA

The SNPlex System assay is equally effective whether you use dried-down or wet gDNA. However, if your experiment requires multiple plates that use the same gDNA or if you plan to use the same gDNA in several experiments, it is convenient to dry-down the gDNA in the plates, which are then ready for use at any time.

Dispensing gDNA into Reaction Plates

To dispense gDNA into reaction plates:

1. Label the reaction plate.



C = Control DNA, NTC = No Template Control, L = Allelic Ladder

For information about proper sample plate layout, refer to "Designing the Sample Plate Layout" on page 3-3.

2. Into each of the control DNA wells, add
   - 1.26 µL 1X TE buffer, pH 8.0
   - 0.74 µL Control DNA (≈37 ng) (from the SNPlex System Assay Control Kit, PN 4349363)

   Note: Control DNA is already fragmented when shipped.

3. Into each of the NTC wells, add 2 µL of 1X TE buffer, pH 8.0.

4. Leave the wells for the allelic ladder empty.

   For information on setting up the allelic ladder wells, refer to "Preparing Samples for Electrophoresis" on page 3-24.

5. Dispense 2 µL of the fragmented gDNA, from step 8 on page 3-7, into the remaining wells of the plate, then briefly centrifuge the plate to ensure that the contents are collected at the bottom of the wells.

6. Allow the plate to air dry for three days in a dark, amplicon-free location. Cover the plate with a lint-free tissue while air-drying.

7. Verify that all the liquid has evaporated.

8. Seal the plates and store at room temperature in the dark until use.

Phosphorylating and Ligating Probes to gDNA (OLA)

For a summary of the steps in the OLA procedure, refer to "Phosphorylating and Ligating Probes to gDNA (OLA)" on page 1-18.

Oligonucleotide Ligation Kit Components

The components in the SNPlex System™ Oligonucleotide Ligation Kit (PN 4362268) are listed below. The kit contains enough reagent for 5,000 reactions.

Component	Storage Temperature (°C)
Oligonucleotide Ligation Master Mix, SNPlex System[a]	4 to 8
dATP (100×), SNPlex System	–15 to –25
Universal Linkers, 48-plex SNPlex System	

a. The Oligonucleotide Ligation Master Mix is shipped frozen. After first use, store at 4 to 8 °C for up to 1 month. Vortex briefly prior to use.

About SNPlex System Ligation Probes

The pooled SNPlex System Ligation Probe Pools that you order arrive in individually labeled tubes. Each tube contains a pool of the following three probe types, mixed together:

- Allele-specific oligo A1 (ASO_{A1})
- Allele-specific oligo A2 (ASO_{A2})
- Locus-specific oligo (LSO)

The universal linkers are delivered in a separate tube.

Preparing the OLA Reactions

To prepare the OLA reactions:

1.	Thaw the following reagents at room temperature: • Universal Linkers, 48-plex SNPlex System • dATP (100×), SNPlex System • SNPlex System Ligation Probes
2.	Thaw the OLA Master Mix at 4 to 8 °C, then invert several times to mix. If a precipitate forms with the OLA Master Mix, place the tube briefly in a heating block set to 37 °C. **Note:** You can store the OLA Master Mix at 4 to 8 °C for up to one month or frozen at –20 °C for up to 1 year.
3.	Vortex, then quick-spin the tubes.

To prepare the OLA reactions: ***(continued)***

4. Prepare an OLA reaction mix by scaling the volumes indicated below to the desired number of OLA reactions.

   You can set up the reactions at room temperature.

   IMPORTANT! Prepare extra volume to account for losses that may occur during pipetting.

Component	Volume per Reaction (µL)	
	Dried gDNA Method	Wet gDNA Method
Nuclease-free water	2.30	0.30
Oligonucleotide Ligation Master Mix SNPlex System	2.50	2.50
Universal Linkers, 48-plex	0.05	0.05
SNPlex System Ligation Probes	0.10	0.10
dATP (100X), SNPlex System	0.05	0.05
Total	5.00	3.00

   Note: Once prepared, you can keep the OLA reaction mix for up to 6 hours at room temperature before use without a loss in performance. After 6 hours, you may store the OLA reaction mix for up to 4 days at 4 °C for later use. You may also prepare a large quantity of the OLA reaction mix and store it at 4 °C or at –20 °C for up to 4 days.

5. Depending on which procedure you have selected, assemble the OLA reaction as described in:
   - "Assembling the OLA Reaction: Dried-Down gDNA" on page 3-11
   - "Assembling the OLA Reaction: Wet gDNA" on page 3-12

Assembling the OLA Reaction: Dried-Down gDNA

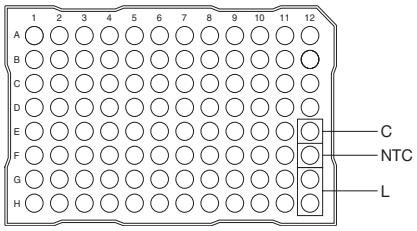
You can set up the reactions at room temperature.

To prepare the OLA reaction when using dried-down gDNA:

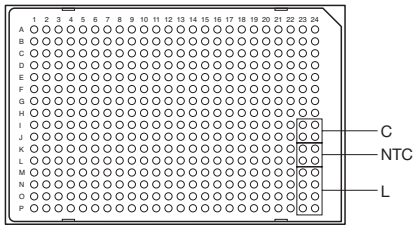
1. Retrieve the reaction plates containing the fragmented, dried gDNA (see "Preparing Genomic DNA" on page 3-6).

   If the plates have not been labeled, label them as shown in the following figures.

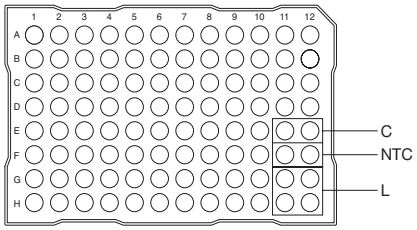
   96-capillary array, 96-well plate

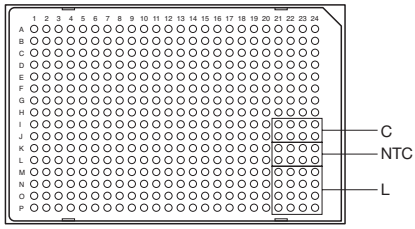
   96-capillary arrays, 384-well plate

   48-capillary array, 96-well plate

   48-capillary array, 384-well plate

   C = Control DNA, NTC = No Template Control, L = Allelic Ladder

   For information about proper sample plate layout, refer to "Designing the Sample Plate Layout" on page 3-3.

2. Pipette 5.0 µL of OLA reaction mix (see step 4 on page 3-10) into each well of the plate.

   IMPORTANT! Do not add reaction mix into the allelic ladder wells.

   For information on setting up the allelic ladder wells, refer to "Preparing Samples for Electrophoresis" on page 3-24.

3. Cover 384-well reaction plates containing the SNPlex OLA reactions with one of the recommended plate covers (see Table 1-3 on page 1-10) and an optical cover compression pad.[a]

   Note: If you are using 96-well plates, use MicroAmp® Full Plate Covers to seal the plate.

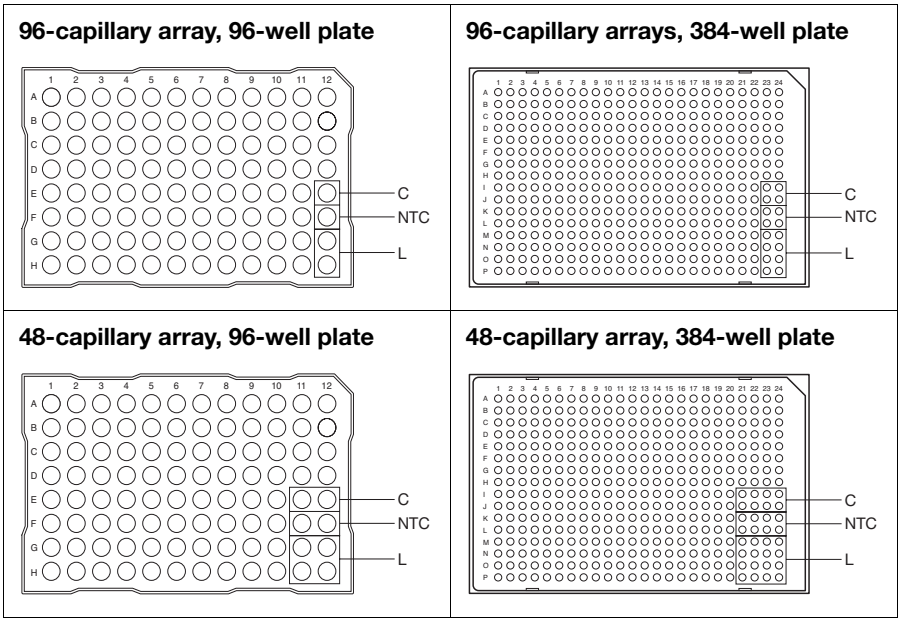
a. IMPORTANT! Applied Biosystems has found that certain plate covers negatively affect the performance of the SNPlex System assay. If you use covers other than the recommended plate covers, test them using the SNPlex™ System Control Set (see Appendix A).

Assembling the OLA Reaction: Wet gDNA

You can set up the reaction at room temperature.

To prepare the OLA reaction when using wet gDNA:

1. Retrieve and label the appropriate number of reaction plates.



C = Control DNA, NTC = No Template Control, L = Allelic Ladder

For information about proper sample plate layout, refer to "Designing the Sample Plate Layout" on page 3-3.

2. Pipette 3.0 µL of OLA reaction mix (see step 4 on page 3-10) into each well of the plate.

   IMPORTANT! Do not add reaction mix into the allelic ladder wells.

   For information on setting up the allelic ladder wells, refer to "Preparing Samples for Electrophoresis" on page 3-24.

3.
   - Into each sample well, add 2.0 µL of gDNA (from step 8 on page 3-7).
   - Into each control well (wells labeled C in diagrams above), add 2.0 µL of control DNA (see step 2 on page 3-8).

4. Cover 384-well reaction plates containing the SNPlex OLA reactions with one of the recommended plate covers (see Table 1-3 on page 1-10) and an optical cover compression pad.[a]

   Note: If you are using 96-well plates, use MicroAmp® Full Plate Covers to seal the plate.

5. Transfer the reaction plates to a thermal cycler.

a. IMPORTANT! Applied Biosystems has found that certain plate covers negatively affect the performance of the SNPlex System assay. If you use covers other than the recommended plate covers, test them using the SNPlex™ System Control Set (see Appendix A).

Running the OLA Reactions on the Thermal Cycler

To thermal-cycle the OLA reactions:

1. If you use an Applied Biosystems thermal cycler, program the thermal cycler as follows. Thermal cycling conditions are the same for 384- and 96-well plates, except for the ramp, as indicated in the following table:

Step	Step Type	Temperature (°C)	Time
1	HOLD	48	30 min
2	HOLD	90	20 min
3	25 Cycles	94	15 sec
		60	30 sec
		51 3% ramp[a]	30 sec
4	HOLD	99	10 min
5	HOLD	4	∞

a. Use a 2% ramp with standard or maximum setting for 96-well plates.

   If you use non-Applied Biosystems thermal cyclers, use the following thermal-cycling parameters. Thermal cycling parameters are identical for 384- and 96-well plates.

Step	Step Type	Temperature (°C)	Time
1	HOLD	48	30 min
2	HOLD	90	20 min
3	25 Cycles	94	15 sec
		57	7 min
4	HOLD	99	10 min
5	HOLD	4	∞

2. When thermal-cycling is complete, remove the OLA reaction plate from the thermal cycler.
3. Briefly spin the OLA reaction plates to collect the liquid in the bottom of the wells.
4. For best results, proceed directly to exonuclease digestion of OLA products, as described in "Purifying Ligated OLA Reaction Products" on page 3-14. Alternatively, you can store the OLA reactions at –20 °C for up to 21 days.

Purifying Ligated OLA Reaction Products

For a summary of the steps in the purification procedure, refer to "Purifying Ligated OLA Reaction Products" on page 1-20.

Purification Kit Components

The components in the SNPlex™ System Purification Kit (PN 4349357) are listed in the table below. The kit contains enough reagent for 5,000 reactions.

Component	Storage Temperature (°C)
Lambda Exonuclease, SNPlex System	–15 to –25
Exonuclease I, SNPlex System	–15 to –25
Exonuclease Buffer, SNPlex System	–15 to –25

Required Materials

Refer to "Required Non-Kit Materials" on page 1-10 for a complete list of vendors and part numbers.

Preparing an Exonuclease Reaction

To prepare an exonuclease reaction:

1. Thaw the Exonuclease Buffer at room temperature.

   If a precipitate forms, place the tube briefly in a heating block set to 37 °C.

2. Vortex, then quick-spin the tubes.

3. Prepare a 2X Exonuclease master mix on ice by scaling the volumes listed below to the desired number of OLA reactions.

   Note: Prepare extra volume to account for losses that may occur during pipetting.

Component	Volume per Reaction (µL)
Nuclease-free water	4.2
Exonuclease Buffer (10X) SNPlex System	0.5
Lambda Exonuclease SNPlex System	0.2
Exonuclease I SNPlex System	0.1
Total	5.0

   Note: Prepare the 2X Exonuclease master mix on ice immediately before use. Applied Biosystems does *not* recommend preparing a large volume of the 2X Exonuclease master mix for later use.

4. Pipette 5 µL of 2X Exonuclease master mix into each well of the OLA reaction plate.

5. Seal the plate with one of the recommended plate covers (see Table 1-3 on page 1-10). If you are using 96-well plates, use MicroAmp® Full Plate Covers to seal the plate.

To prepare an exonuclease reaction: ***(continued)***

<table>
<tr><td>6.</td><td>Vortex the plates and spin to collect liquid in the bottom of the wells.</td></tr>
<tr><td>7.</td><td>Program the thermal cycler:
<table>
<tr><th>Step</th><th>Step Type</th><th>Temperature (°C)</th><th>Time</th></tr>
<tr><td>1</td><td>HOLD</td><td>37</td><td>90 min</td></tr>
<tr><td>2</td><td>HOLD</td><td>80</td><td>10 min</td></tr>
<tr><td>3</td><td>HOLD</td><td>4</td><td>∞</td></tr>
</table></td></tr>
<tr><td>8.</td><td>Transfer the reaction plates to the thermal cycler, and start the program.</td></tr>
<tr><td>9.</td><td>After thermal-cycling is complete, spin to collect liquid in the bottom of the wells.</td></tr>
<tr><td>10.</td><td>Add 15 µL of nuclease-free water to each well, mix, then spin down.</td></tr>
<tr><td>11.</td><td>Process the enzyme-digested OLA reaction products. To use the OLA reaction products:
• Immediately – Proceed to “Performing PCR” on page 3-16.
• Within 21 days – Store at –20 °C.

Note: For storage, seal the plates with one of the recommended plate covers (see Table 1-3 on page 1-10).

Note: For best results, use the OLA reaction products immediately.</td></tr>
</table>

Performing PCR

For a summary of the steps in the amplification procedure, refer to "PCR Amplifying Ligated OLA Reaction Products" on page 1-21.

Amplification Kit Components

The components in the SNPlex™ System Amplification Kit (PN 4349358) are listed in the table below. The kit contains enough reagent for 5,000 reactions.

Component	Storage Temperature (°C)
Amplification Master Mix (2X) SNPlex System	2 to 8
Amplification Primers (20X) SNPlex System	–15 to –25

Required Materials

Refer to "Required Non-Kit Materials" on page 1-10 for a complete list of vendors and part numbers.

Preparing the PCR Master Mix

To prepare the PCR master mix:

1. Thaw the Amplification Primers.
2. Vortex, then quick-spin the tube.
3. Prepare a PCR master mix by scaling the volumes listed below to the desired number of PCR reactions.

   Note: Prepare extra volume to account for losses that may occur during pipetting.

Component	Volume per Reaction (µL)
Nuclease-free water	2.4
Amplification Master Mix (2X) SNPlex System	5.0
Amplification Primers (20X) SNPlex System	0.5
Total Volume	7.9

Assembling and Running the PCR Reaction

To assemble and run the PCR reaction:

1. Into each well of a 384- or 96-well plate, dispense:
   - 7.9 µL PCR master mix
   - 2.1. µL diluted OLA reaction product (see step 10 on page 3-15)

To assemble and run the PCR reaction: ***(continued)***

<table>
<tr><td>2.</td><td>Cover 384-well reaction plates containing the SNPlex OLA reactions with one of the recommended plate covers (see Table 1-3 on page 1-10) and an optical cover compression pad.[a]

Note: If you are using 96-well plates, use MicroAmp® Full Plate Covers to seal the plate.</td></tr>
<tr><td>3.</td><td>Program the thermal cycler:
<table>
<tr><th>Step</th><th>Step Type</th><th>Temperature (°C)</th><th>Time</th></tr>
<tr><td>1</td><td>HOLD</td><td>95</td><td>10 min</td></tr>
<tr><td rowspan="2">2</td><td rowspan="2">30 cycles</td><td>95</td><td>15 sec</td></tr>
<tr><td>63</td><td>1 min</td></tr>
<tr><td>3</td><td>HOLD</td><td>4</td><td>∞</td></tr>
</table></td></tr>
<tr><td>4.</td><td>Transfer the reaction plates to the thermal cycler and start the program.</td></tr>
<tr><td>5.</td><td>When thermal cycling is complete, remove the reaction plates.</td></tr>
<tr><td>6.</td><td>If you use the PCR reaction products:
• Immediately – Proceed to “Hybridizing PCR Products to ZipChute Probes” on page 3-18.
• Within 24 hours – Store at 4 °C.
• Within 35 days – Store at –20 °C.</td></tr>
</table>

a. IMPORTANT! Applied Biosystems has found that certain plate covers negatively affect the performance of the SNPlex System assay. If you use covers other than the recommended plate covers, test them using the SNPlex™ System Control Set (see Appendix A).

Hybridizing PCR Products to ZipChute Probes

For a summary of the steps in the ZipChute hybridization procedure, refer to "Hybridizing PCR Products to ZipChute Probes and Performing Electrophoresis" on page 1-22.

Reagents Required for Hybridization

The reagents required to complete the hybridization process are listed below. Each reagent or kit contains enough volume for 5,000 reactions.

Component	Storage Temperature (°C)
SNPlex System Hybridization Plates, 384-well or SNPlex System Hybridization Plates, 96-well	Ambient
Hybridization Wash Buffer SNPlex System	Ambient
Hybridization Binding Buffer SNPlex System	Ambient
ZipChute Dilution Buffer SNPlex System	Ambient
SNPlex™ System ZipChute Kit, 48-plex[a] • Denaturant SNPlex System • ZipChute Mix, 48-plex SNPlex System • Positive Hybridization Controls SNPlex System	–15 to –25

a. Avoid exposure to light and minimize freeze-thaw cycles.

Required Materials

Refer to "Required Non-Kit Materials" on page 1-10 for a complete list of vendors and part numbers.

Preparing the Hybridization Plates

To prepare the hybridization plates:

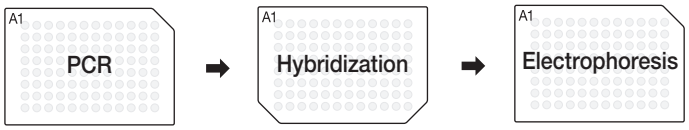
1.	Remove the PCR reaction plates from storage (see step 6 on page 3-17).
2.	Vortex, then briefly spin the PCR reaction plates to collect the liquid in the bottom of the wells.
3.	Label the SNPlex Hybridization Plates, making sure well A-1 is at the top left corner.
4.	Wash the wells of the SNPlex Hybridization Plates three times with 100 µL Wash Buffer diluted 1:10. **Note:** Dilute the Wash Buffer 1:10 with deionized water.

To prepare the hybridization plates: *(continued)*

5.	Briefly shake the plates upside down on a clean paper towel. **IMPORTANT!** For this and all subsequent washing steps, all excess liquid must be removed from the plate before adding new reagents. However, keeping the plates empty for extended periods of time negatively affects the performance of the SNPlex System assay.
6.	Prepare the Binding Buffer with the Positive Hybridization Control for the desired number of hybridization reactions. For each reaction, you need • 17.491 µL of undiluted Binding Buffer • 0.009 µL of Positive Hybridization Control **Note:** Prepare extra volume to account for losses that may occur during pipetting.

Binding PCR Products to the Hybridization Plate

To bind the PCR product to the hybridization plate:

1.	Add 17.5 µL of Binding Buffer containing Positive Hybridization Control to the SNPlex Hybridization Plate.
2.	Transfer 1.5 µL of each well containing the PCR reaction product into each well of the SNPlex Hybridization Plate and mix. **Note:** The notches on the plates do not always line up. Make sure you orient the plates with well A-1 at the upper left corner when transfering samples between plates. 
3.	Cover the SNPlex Hybridization Plate with one of the recommended plate covers (see Table 1-3 on page 1-10).
4.	Incubate for 60 min at room temperature on a rotary shaker set to high.
5.	Briefly spin the hybridization plates to collect the liquid in the bottom of the wells.

Isolating Biotinylated Strands on the Hybridization Plate

To isolate the biotinylated strand on the hybridization plates:

1.	Uncover the SNPlex Hybridization Plates.
2.	Remove the supernatant from each well.
3.	Wash each well three times with 100 µL Wash Buffer diluted 1:10. **Note:** Dilute the Wash Buffer 1:10 with deionized water.

To isolate the biotinylated strand on the hybridization plates: *(continued)*

4.	Briefly shake the plates upside down on a clean paper towel. **IMPORTANT!** All excess liquid must be removed from the plate before adding new reagents. However, keeping the plates empty for extended periods of time negatively affects the performance of the SNPlex System assay.
5.	Add 50 µL of 0.1 N NaOH, then cover the plate with one of the recommended plate covers (see Table 1-3 on page 1-10). **Note:** Applied Biosystems recommends that you prepare the 0.1N sodium hydroxide solution fresh every 4 weeks.
6.	Incubate for 5 to 30 min at room temperature on a rotary shaker.
7.	Carefully remove the supernatant from each well, then wash each well five times with 100 µL of Wash Buffer diluted 1:10. **Note:** Dilute the Wash Buffer 1:10 with deionized water.
8.	Briefly shake the plates upside down on a clean paper towel. **IMPORTANT!** All excess liquid must be removed from the plate before adding new reagents. However, keeping the plates empty for extended periods of time negatively affects the performance of the SNPlex System assay.

Hybridizing the ZipChute Probes

WARNING CHEMICAL HAZARD. Zipchute Dilution Buffer, SNPlex System. Exposure causes eye irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Denaturant, SNPlex System. Exposure causes eye, skin, and respiratory tract irritation. Denaturant, SNPlex System is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To hybridize the ZipChutes Probes:

1. Equilibrate the oven to 37 °C.

2. Prepare a hybridization master mix by scaling the volumes listed below to the desired number of samples. Prepare extra volume to account for losses that may occur during pipetting.

Component	Volume per Reaction (µL)
ZipChute Mix, 48-plex SNPlex System	0.05
Denaturant, SNPlex System	11.25
ZipChute Dilution Buffer, SNPlex System	13.70
Total	25.00

   Note: Once prepared, you can keep the hybridization master mix at room temperature for at least 2 hours without a loss in performance. After 2 hours the remaining hybridization master mix may be stored covered in the dark at 4 °C for up to 4 days for later use.
   You may prepare a large quantity of hybridization master mix and store it covered in the dark at 4 °C for up to 4 days.

3. Add 25 µL of the hybridization master mix to each well.

4. Cover the plate with one of the recommended plate covers (see Table 1-3 on page 1-10).

5. Incubate the plates for 60 min at 37 °C on a rotary shaker.

   Note: During incubation, avoid exposure to direct light.

   Note: To avoid possible overheating, do not place the plate directly on the floor of the oven.

Eluting ZipChute Probes

For a summary of the steps in the purification procedure, refer to "Hybridizing PCR Products to ZipChute Probes and Performing Electrophoresis" on page 1-22.

Standards Kit Components

The components included in the SNPlex System Assay Standards Kit (PN 4349351) are listed in the table below. The kit contains enough reagent for 5,000 reactions.

Component	Storage Temperature (°C)
Size Standard, 48-plex SNPlex System[a]	–15 to –25
Sample Loading Reagent, SNPlex System	–15 to –25
Allelic Ladder, 48-plex SNPlex System[b]	–15 to –25

a. After the reagent is opened, store at 4 °C and minimize exposure to light.
b. Minimize exposure to light.

IMPORTANT! The effectiveness of the Allelic Ladder and Size Standard declines with increasing freeze-thaw cycles. Make aliquots as necessary.

Required Materials

Refer to "Required Non-Kit Materials" on page 1-10 for a complete list of vendors and part numbers.

Preparing the Sample Loading Mix

WARNING CHEMICAL HAZARD. Sample Loading Reagent, SNPlex System. Exposure causes eye, skin, and respiratory tract irritation. SNPlex Sample Loading Reagent is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the sample loading mix:

1. Thaw the Size Standard, Allelic Ladder 48-plex, and Sample Loading Reagent at room temperature.

   If precipitates form, place the tubes briefly in a heating block set to 37 °C.

2. Vortex, then quick-spin the tubes.

3. Prepare a sample loading mix by scaling the volumes listed below to the desired number of samples.

   Note: Prepare extra volume to account for losses that may occur during pipetting.

   Note: Prepare fresh sample loading mix daily.

Component	Volume per Reaction (µL)
Size Standard, 48-plex SNPlex System	0.6
Sample Loading Reagent, SNPlex System	16.9
Total Volume	17.5

Eluting the ZipChute Probes

To elute the ZipChute probes:

1.	After the 60-min incubation period (see page 3-21), remove the supernatant from the hybridization plates. **IMPORTANT!** For best results, do not let plates sit at room temperature for extended periods of time before removing supernatant.
2.	Wash each well four times with 100 µL Wash Buffer diluted 1:10. **Note:** Dilute the 10× Wash Buffer 1:10 with deionized water. **IMPORTANT!** The ZipChute probes may be stripped off the plate under the following conditions: • Rapid aspiration of the ZipChute Mix or Wash Buffer supernatant when using a plate washer • Contact between the plate washer tips and the well surfaces Applied Biosystems recommends that you set the aspiration tip depth so that 15 to 20 µL of Wash Buffer remains in each well after each aspiration, preventing the tips from touching the bottom of the wells.[a]
3.	**IMPORTANT!** For consistent results, after the last wash, spin the plate ***upside down*** at 1000 rpm for 60 sec on a stack of clean paper towels to remove all remaining buffer.
4.	Immediately add 17.5 µL of Sample Loading Mix containing size standard to each well.
5.	Cover the plate containing the size standard with one of the recommended plate covers (see Table 1-3 on page 1-10).
6.	Incubate the plate in a 37 °C oven for 30 min on a rotary shaker. **Note:** To avoid possible overheating, do not place the plate directly on the floor of the oven.

a. For more information about configuring plate washers, refer to the *SNPlex™ Genotyping System 48-plex General Automation Getting Started Guide* or the *SNPlex™ Genotyping System 48-plex Automation Guide Automating PCR Using the Tomtec Quadra 3 Getting Started Guide*.

Preparing Samples for Electrophoresis

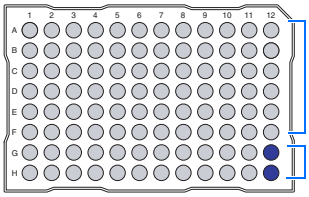
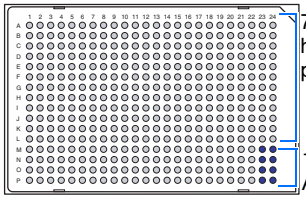
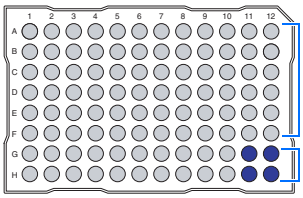
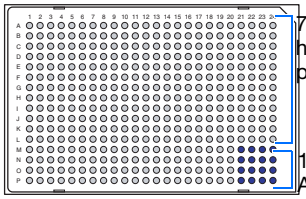
To dispense the allelic ladder:

1.	Remove the hybridization plates from the oven and mix.
2.	Briefly spin the plates to collect the liquid at the bottom of the wells.
3.	Label a new reaction plate.
4.	• If using 384-well plates, transfer 7.5 µL from each well into the wells of the new plate. • If using 96-well plates, transfer 10 µL from each well into the wells of the new plate.

To dispense the allelic ladder: ***(continued)***

5. Load the Allelic Ladder.

Note: The Allelic Ladder is part of the SNPlex System Standards Kit.

If using 96-well plates, pipette 1.25 µL of Allelic Ladder, 48-plex plus 10 µL of Sample Loading Mix[a] into the wells designated below.	If using 384-well plates, pipette 1.0 µL of Allelic Ladder, 48-plex plus 7.5 µL of Sample Loading Mix[a] into the wells designated below.
96-capillary array, 96-well plate  10 µL from hybridization plate 1.25 µL Allelic Ladder 10 µL Sample Loading Mix	**96-capillary arrays, 384-well plate**  7.5 µL from hybridization plate 1 µL Allelic Ladder 7.5 µL Sample Loading Mix
48-capillary array, 96-well plate  10 µL from hybridization plate 1.25 µL Allelic Ladder 10 µL Sample Loading Mix	**48-capillary array, 384-well plate**  7.5 µL from hybridization plate 1 µL Allelic Ladder 7.5 µL Sample Loading Mix

a. See "Preparing the Sample Loading Mix" on page 3-22.

Note: After transferring the sample loading mix, make sure there are no air bubbles trapped at the bottom of the wells. If there are, briefly spin the plate.

Note: For information about proper sample plate layout, refer to "Designing the Sample Plate Layout" on page 3-3.

IMPORTANT! If you are not going to immediately use the plates for analysis, seal the plates, and store at –20 °C.

Note: Consider the plate seal options for use with the 3730 and 3730*xl* instruments. While both septa andheat seal film are available, the septa do not provide an air-tight seal. Some gradual signal loss occurs over time when using the septa. If the SNPlex plates will remain on the instrument in excess of 12 hours, Applied Biosystems recommends using the pierceable heat seal option (Heat Seal film, PN 4337570). Be aware that after the heat seal is pierced by the instrument for sample injection, the seal is no longer intact.

Creating Results Groups and Plate Records

Starting Data Collection Software

To start the 3730 Analyzer Data Collection Software:

1.	Select **Start** > **Programs** > **Applied Biosystems** > **Data Collection** > **Run 3730 Data Collection** v2.0 or higher.
2.	Wait as the Service Console dialog box starts the applications of the data collection software.
3.	When all applications are running, the Data Collection Viewer opens.

About Results Groups

Results Groups allow you to specify autoanalysis settings, designate a data storage location, and specify naming conventions for sample files and run folders.

By including "Plate Quadrant" as a parameter for naming run folders, the Data Collection software automatically generates separate run folders for each plate quadrant. Because the sample plate is set up so that each plate quadrant corresponds to a probe pool, the data for each probe pool is stored in a separate folder.

If you use a 48-capillary array, including "Run Sequence Number" or "Run Name" as a parameter for naming run folders enables the Data Collection software to generate a separate folder for each run required to complete a sample plate (see "Designing the Sample Plate Layout" on page 3-3).

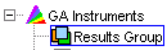
For example, if you use a 48-capillary instrument, two runs are required to run a single 96-well plate or one quadrant of a 384-well plate (total of eight runs for a 384-well plate). Similarly, if you use a 96-capillary instrument, a single run is required for a 96-well plate or one quadrant of a 384-well plate (total of four runs for a 384-well plate).

Applied Biosystems recommends that you use the above Results Groups conventions in order to allow separate sizing bin adjustments to be applied to individual runs, even if they originate from the same probe pool (quadrant). In the latter case, you can cluster the runs individually or together (as a project).

IMPORTANT! For GeneMapper® software to correctly process SNPlex System data, run folder naming conventions and sample plate layout (explained in "Designing the Sample Plate Layout" on page 3-3) must correspond to each other.

Setting Up Results Groups

To set up results groups for SNPlex System experiments:

1.	In the Data Collection software, double-click **Results Group** to open the Results Group Editor. 
2.	Select the **Naming** tab.

To set up results groups for SNPlex System experiments: ***(continued)***

3.	Complete the information in the tab as shown in Figure 3-2 and Table 3-1. **Note:** When you create a new results group, the Data Collection software displays a single drop-down box under each Format section. Each time you make a selection (as specified in the table below), the software adds a drop-down box.

Figure 3-2 shows the Results Group Editor for a sample Results Group used with 3730 instruments running a 48-capillary array. Note that for instruments running 96-capillary arrays, the Run Sequence Number is unnecessary.

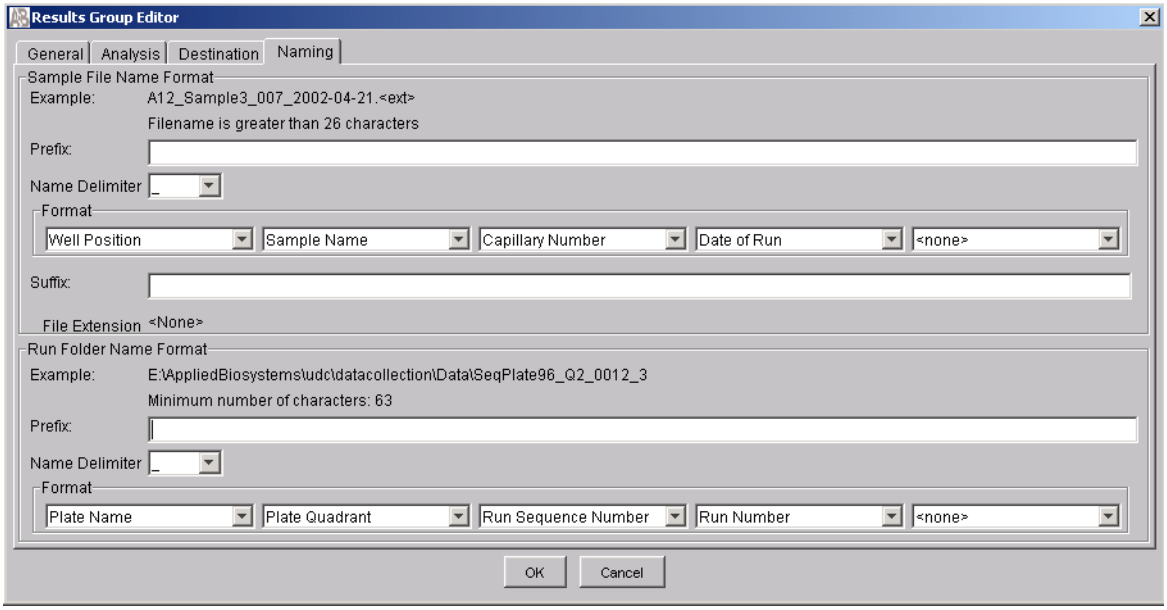


Figure 3-2 Results Group settings for 3730 instruments running 48-capillary arrays

Table 3-1 Suggested minimum sample file and run folder parameters for SNPlex System results groups

Parameter	Comment
Sample File Name	In the Format section under Sample File Name Format, select ...
Well Position	**Well Position**
Sample Name	**Sample Name**
Capillary Number	**Capillary Number**
Run Folder Name	In the Format section under Run Folder Name Format, select ...
Plate Name	**Plate Name**
Plate Quadrant	**Plate Quadrant**

Table 3-1 Suggested minimum sample file and run folder parameters for SNPlex System results groups *(continued)*

Parameter	Comment
Run Sequence Number[a]	**Run Sequence Number** **Note:** If this parameter is selected, the Data Collection software adds a four-digit number to the run folder name. The number is incremented with every run on the instrument. This option is highly recommended for the 3730 instrument when running 48-capillary arrays. It is not necessary when running 96-capillary arrays.
Run Name	An alternative to Run Sequence Number. If this parameter is selected, the Data Collection software appends a text string, such as Run_InstrumentName_Date_Time_RunSequence Number, to the run folder name.
Run Number	**Run Number**

a. Preferred over Run Name.

About Plate Records

A plate record is similar to a sample sheet or an injection list that you may have used with some Applied Biosystems instruments. Plate records are data tables in the instrument database that store information about the plates and the samples they contain.

Some plate record fields that are required for 3730/3730*xl* analyzer operation and sample file generation must be completed before a run. Depending on the needs of your laboratory, you can either:

- Complete the plate record manually, adding information at the appropriate time in the workflow.
  or
- Partially or fully automate the plate record creation process by importing information from LIMS or text editor-generated files.

Creating Plate Records

There are several ways to create plate records. Figure 3-3 illustrates three possible methods: manual, partially automated, and fully automated.

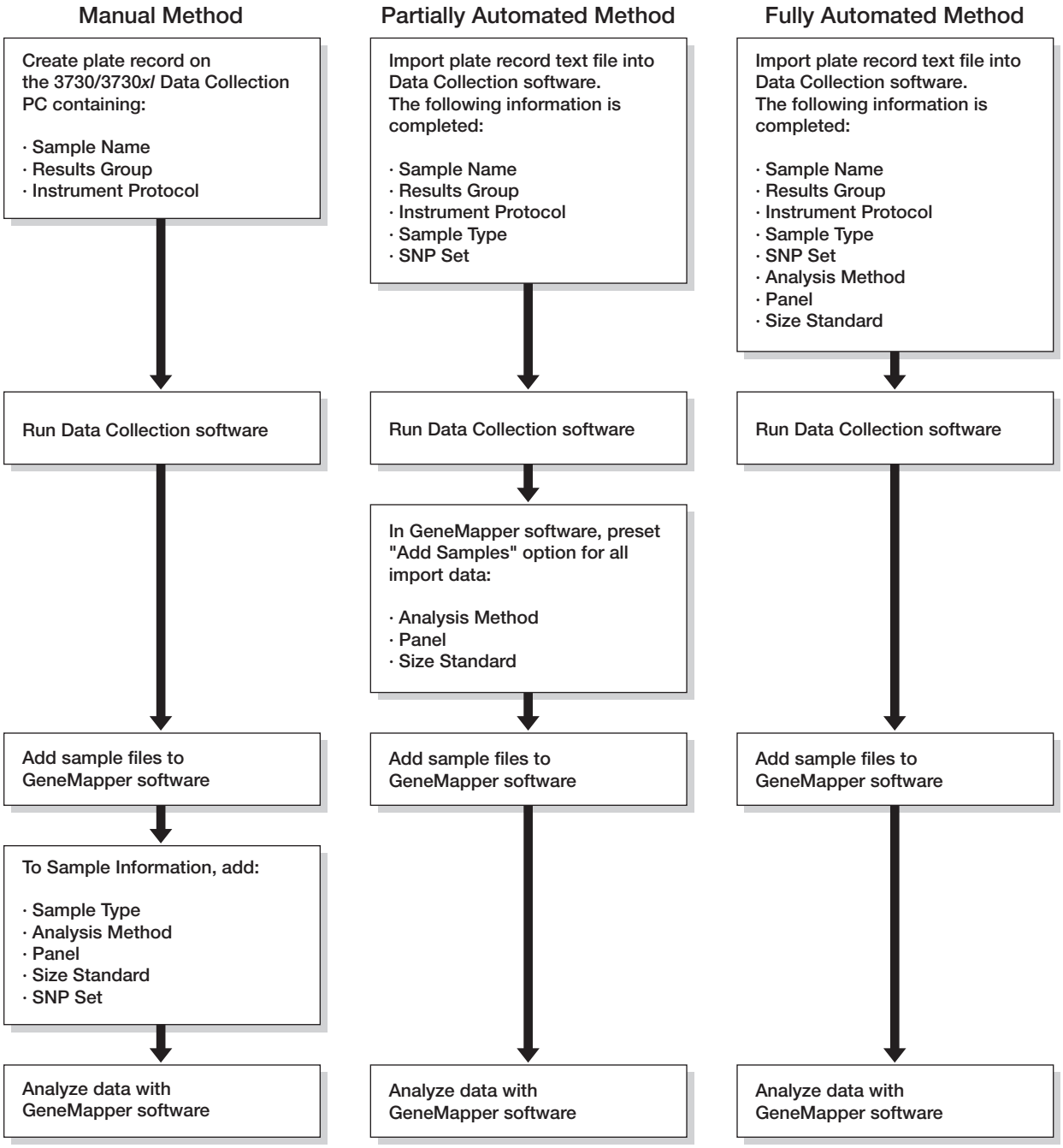


Figure 3-3 Three possible methods for creating plate records

Using GeneMapper Software

Another consideration you need to make when creating plate records is which GeneMapper software application you intend to use.

When GeneMapper software is installed on a computer that has Data Collection software, two applications are available (in the Automated Processing tab of the Results Group Editor):

- **GeneMapper-Generic** – Generates sample files but does not perform autoanalysis.
- **GeneMapper-<*Instrument Name*>** – Performs autoanalysis.

Table 3-2 indicates the required fields for creating plate records using each application.

Table 3-2 Minimum required fields for fragment analysis data collection

Field	GeneMapper-Generic	GeneMapper-<*Instrument Name*>
3730/3730xl Analyzer Fields		
Sample Name	required	required
Results Group	required	required
Instrument Protocol	required	required
GeneMapper Software Fields		
Size Standard	optional	required
Analysis Method	optional	required
SNP Set	optional	required
Panel	optional	required
Sample Type	optional	required

When creating plate records, note that

- You must complete the required 3730/3730xl fields before a run, regardless of which GeneMapper software application you are using to analyze the data.
- If you run the GeneMapper-Generic application, you can complete the GeneMapper software fields after the electrophoresis run, but before analyzing the data. Refer to Chapter 4 for information on setting up GeneMapper software.

  Note: You cannot analyze the samples in GeneMapper software unless these fields are completed.

- If you run the GeneMapper-<*Instrument Name*> application, you also need to complete the GeneMapper software fields before the electrophoresis run.
  - You can preset GeneMapper software to automatically apply the Analysis Method, Panel, and Size Standard whenever sample files are imported, as explained in "Importing SNPlex System Data into GeneMapper Software" on page 4-6.
  - You can import SNP Sets from assay information files, as explained in "Importing the AIF" on page 4-5.
  - When adding Sample Type and SNP Set information to a plate record, enter them in such a way that the data can be readily analyzed by GeneMapper software without you having to edit the plate record. Additionally, these fields must be entered exactly as they are defined in GeneMapper software.
- The most convenient way to create plate records is to import appropriately formatted text files that have been generated by a text editor or by a LIMS sysem. The simplest way to get started is to export a working plate record using the Data Collection software, then use it as a template to develop a plate record generation tool.
- Plate records exported by the Data Collection software contain additional header information, including Container Name, Plate ID, Description, ContainerType, AppType, Owner, Operator, PlateSealing, and SchedulingPref. Again, the simplest way to define these fields correctly is to use a working plate record as a guide.

Creating Plate Records by Importing Formatted Text Files

Applied Biosystems recommends using a partially automated method to generate plate records (see Figure 3-3). Such a method helps eliminate problems arising from data-entry errors and can also greatly reduce the time spent setting up plate records.

To set up plate records by importing text files:

1.	In the Data Collection software, open the **Plate Manager**.
2.	Click **Import**, then navigate to the text file that you want to import.
3.	Select the file that you want to import, then click **Open**. The Data Collection software imports the contents of the file into a new plate record, then displays a confirmation message if the import is successful. If you set up your text file as recommended, the Sample Name, Results Group, Instrument Protocol, Sample Type, and SNP Set fields are complete at this point. If you have set up the Add Samples options in GeneMapper software ("Setting Analysis Method, Size Standard, and Panel Automatically" on page 4-7), the Analysis Method, Size Standard, and Panel fields will be completed automatically when the sample files are imported into GeneMapper software. **IMPORTANT!** For GeneMapper software to recognize the SNP set information, you must have imported the assay information file into GeneMapper software ("Importing AIFs" on page 4-5).

Creating Plate Records Manually

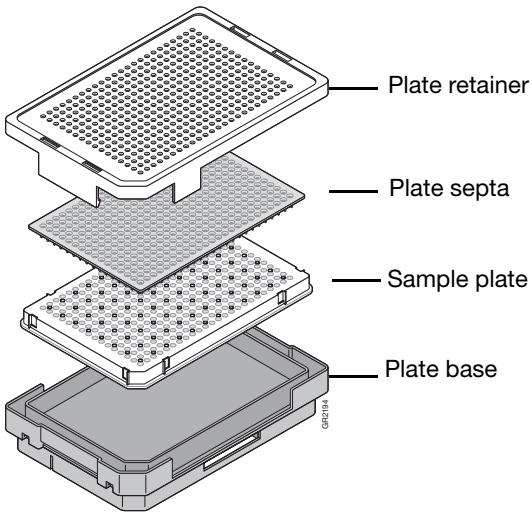
To create the plate record manually:

1.	In the tree pane of the Data Collection Software, double-click **GA Instruments** > **ga3730** or **ga3730*xl*** > <***Instrument Name***> > **Run Scheduler**.
2.	In the Add Plate field of the Run Scheduler view, enter or scan the bar code of a plate that you want to run, then press **Enter**.
3.	In the Select an Option dialog box, click **Yes**.
4.	In the New Plate dialog box, update the following fields: • **ID (Barcode)** – Scan or enter the barcode for the plate you want to run. • **Name** – Enter a name for the plate. • **Description** – Enter a description for the plate record (optional) • **Application** – Select **GeneMapper-Generic** • **Plate setup** – Select **384-Well** or **96-Well** • **Plate sealing** – Select **Septa** or **Heat Seal** (if using heat-sealed plates) • **Owner name** – Enter your owner name • **Operator name** – Enter your operator name
5.	Click **OK**.
6.	In the Plate Editor dialog box, for each row of the plate record table, enter: a. A sample name b. Comments for each well of the plate (optional)
7.	For each row of the Plate Record table: a. Select or create a results group. b. For the instrument protocol, pair the Run Module (HTSNP36_POP7_V2) with Dye Set S. **Note:** Refer to "Importing SNPlex System Files into the Data Collection Software" on page 2-3 if the run module and dye set are not available on your system.
8.	In the Description field, enter a description of the plate record (optional).
9.	Click **OK**. The data collection software saves the plate record to the database.
10.	If running more than one plate, repeat steps 3 to 9. **Note:** At this point you have specified only the Sample Name, Results Group, and Instrument Protocol fields, as shown in the manual workflow in Figure 3-3 on page 3-29. You must specify the rest of the information in the plate record (specifically, in the GeneMapper Sample Table after adding sample files) before you can analyze the data using GeneMapper software.

Loading and Running the Sample Plates

Plate Assembly

Assemble the plates for loading onto the 3730/3730*xl* analyzer. The 384-well plate assembly (shown below) is similar to the 96-well plate assembly (use sample plates, plate septa, and plate retainers for the 96-well format).



Required Materials

Refer to "Required Non-Kit Materials" on page 1-10 for a complete list of vendors and part numbers.

Loading Sample Plates

To load the plates in the stacker:

1.	Pull open the stacker drawer. The stacker light flashes green.
2.	Open the metal door of the In-Stacker tower.
3.	Place the plates in the stacker (16 maximum). The bottom plate runs first. **IMPORTANT!** Ensure that the plate assembly fits flat in the stacker and that plate retainer clips are properly seated in the base.
4.	Close the metal In-Stacker tower door.
5.	Close the stacker drawer.

Prerequisites

If you are using Data Collection v2.0, verify that the default prebatch file has been replaced with the SNPlex System prebatch file.

Note: If you are using Data Collection v3.0, there is no specific prebatch file and you may disregard the following paragraph.

Double-click **PrebatchModule.txt** (typically in E:\AppliedBiosystems\UDC\DataCollection\SupportFiles\ga3730\Service Modules). If the first line of the file is not //SNPlex v2.0 Prebatch, refer to "Replacing the PrebatchModule. txt File" on page 2-3 for more information.

Running the Plates

To run the plates:

1.	In the tree pane of the Data Collection Software, double-click **GA Instruments** > **ga3730** or **ga3730*xl*** > <***Instrument Name***> > **Run Scheduler**.
2.	In the Input Stack group box of the Run Scheduler view, click **Search**, then click **Find All**.
3.	Select the plate record, then click **Add**.
4.	Click the green arrow in the toolbar to begin the run. **Note:** As part of the prebatch function, the instrument oven heats to temperature before the run begins. As the data is collected, you can view it in the Array Viewer.

Analyzing Data Using GeneMapper Software

4

This chapter covers:

Overview

Figure 4-1 summarizes the process of analyzing SNPlex™ System data with GeneMapper® software v3.7.

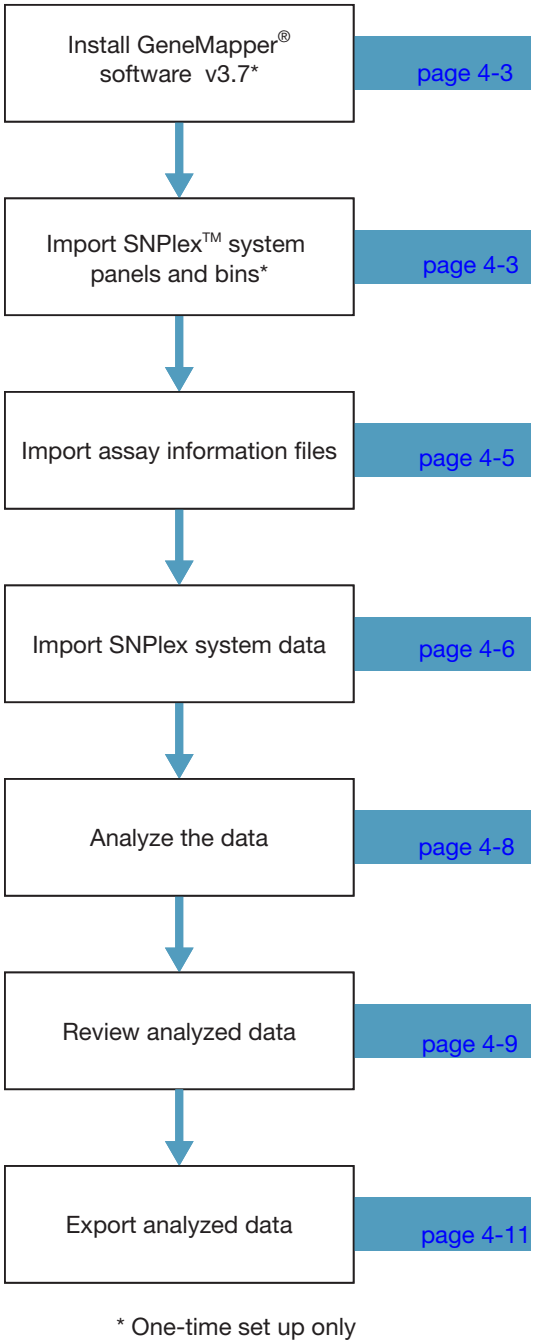


Figure 4-1 Analyzing SNPlex System data with GeneMapper software v3.7

Installing GeneMapper Software v3.7

Install the GeneMapper Software v3.7 according to instructions provided in the GeneMapper® Software v.3.7 Installation Guide (PN 4359289).

Importing SNPlex System Panels and Bins

About SNPlex System Panels and Bins

GeneMapper software uses the same analysis parameters for all 48-plex SNPlex System experiments. All the parameter files required to perform analysis of the SNPlex System assay chemistry by GeneMapper software are installed on your computer when you install GeneMapper software v3.7. (See Table 4-1 on page 4-3.) Importing the parameter files into GeneMapper software is a one-time setup step.

Note: Check for updates on the Applied Biosystems Web site at

http://www.appliedbiosystems.com/support/software

Table 4-1 Parameter files for analyzing SNPlex System data on GeneMapper software v3.7

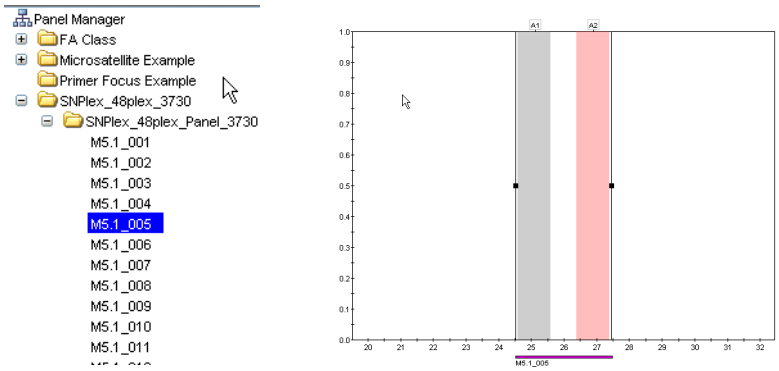
File Name	File Name as Displayed After Import into GeneMapper Software	Description
SNPlex_48plex_3730_Bins.txt	SNPlex_48plex_Bin_3730	Contains bins for SNPlex System allelic ladder. This file, along with the panel file, contains information about the expected sizes of the allelic ladder. **IMPORTANT!** Do not edit this file.
SNPlex_48plex_3730_Panels.txt	SNPlex_48plex_Panel_3730	Contains panels for SNPlex System allelic ladder. This file, along with the bin file, contains information about the expected sizes of the allelic ladder. **IMPORTANT!** Do not edit this file.

Importing SNPlex System Panels and Bins

To import SNPlex System panels and bins:

1.	Start the GeneMapper software v3.7.
2.	Access the Panel Manager. a. Select **Tools > Panel Manager** (**Ctrl**+**J**). b. Click  (Panel Manager).

To import SNPlex System panels and bins: *(continued)*

3.	Import the files. a. Import the panels: Click Panel Manager, click **File > Import Panels** (**Ctrl**+**M**), navigate to the GeneMapper Files folder on the SNPlex System 48-plex Support Files CD, then select **SNPlex_48plex_3730_Panels.txt**. b. Import the bins: Click SNPlex_48plex_3730, click **File > Import Bin Set** (**Ctrl**+**Shift**+**B**), navigate to the GeneMapper Files folder on the SNPlex System 48-plex Support Files CD, then select **SNPlex_48plex_3730_Bins.txt**. **Note:** If, when you try to import the files, the GeneMapper software displays an error message that tells you the settings already exist, override the old settings.
4.	Click **OK** to close the Panel Manager.
5.	Confirm that the SNPlex System panels and bins were imported properly. In the Panel Manager, a. Select, then double-click **SNPlex_48plex_3730**. b. Select, then double-click **SNPlex_48plex_Panel_3730**. c. Select an individual marker, for example, **M5.1_005**.  If the import was successful, two bins (one gray and one pink) are displayed. d. If you do not see the bins, check if the SNPlex System bin set is selected in the bins drop-down list. e. If you do not see the bins, reimport the bin set.

Importing the AIF

About Assay Information Files for the SNPlex System

If your plate records are set up in Data Collection software to include sample names and SNP sets (typically imported from a text file, as recommended in "Creating Results Groups and Plate Records" on page 3-26), you must import the Assay Information File (AIF) into GeneMapper software before you import the SNPlex System sample files (*.fsa). Doing so ensures that GeneMapper software reads the sample names and SNP sets from the plate record.

IMPORTANT! If you are using the SNPlex_Model_3730 analysis method, or any method that uses the Model clustering algorithm or any method that uses analyses by project, you must import the AIF file into GeneMapper software. If you attempt to run the analysis without importing the AIF, you will lose information about markers that are associated with SNPs. (The software assumes that all markers are associated with SNPs.) For more information about analysis methods, refer to Appendix B.

Each SNPlex System probe pool order is accompanied by a SNPlex Genotyping System Ligation Probes CD, which contains the files listed in Table 4-2.

Table 4-2 Files in the SNPlex Genotyping System Ligation Probes CD

File	Description
SNPlex_*nnnnnnnn_nnnnnnn*.xml where *nnnnnnnn_nnnnnnn* is the design ID.	Assay information file that contains information about the probe pool, including SNP set names. Do not modify this file.
ablogo.gif	Applied Biosystems logo file.
aiftypes_v1_1.xsd	Defines the structure of AIF types. Do not modify this file.
Probes_Insert.DOC	Limited license for the SNPlex System ASO/LSO probe sets.
SNPlexAIF_v1_1.xsd	Defines the structure for the AIF file. Do not modify this file.
SNPlexStylesheet_v1_0.xslt	Contains instructions for displaying the SNPlex System data sheet. Do not modify this file.

Importing AIFs

To import AIFs into GeneMapper software:

1.	Select **Tools > GeneMapper Manager**.
2.	In the SNP Sets tab, click **Import**.
3.	Insert the SNPlex Genotyping System Ligation Probes CD , then select the AIF (indicated by SNPlex_*nnnnnnn_nnnnnnn*.xml). When the import is complete, GeneMapper software displays a number of new SNP sets, each corresponding to a single SNPlex System ligation probe pool. The name of each pool is identical to the name on the tube label.

Importing SNPlex System Data into GeneMapper Software

The data-import process consists of two steps:

- Completing required plate record fields.
- Importing SNPlex System sample files.

Required Fields

The following plate record fields must be completed before GeneMapper software can analyze data: Sample Name, Sample Type, SNP Set, Analysis Method, Panel, and Size Standard. Of these fields:

- Sample Name and Sample Type are completed in the Data Collection software prior to the electrophoresis run. These fields can be completed manually or imported from formatted text files (see "Creating Results Groups and Plate Records" on page 3-26).
- SNP Set names can be imported from the AIF into GeneMapper software. The SNP Set names in the Data Collection plate record must match those in the GeneMapper file.

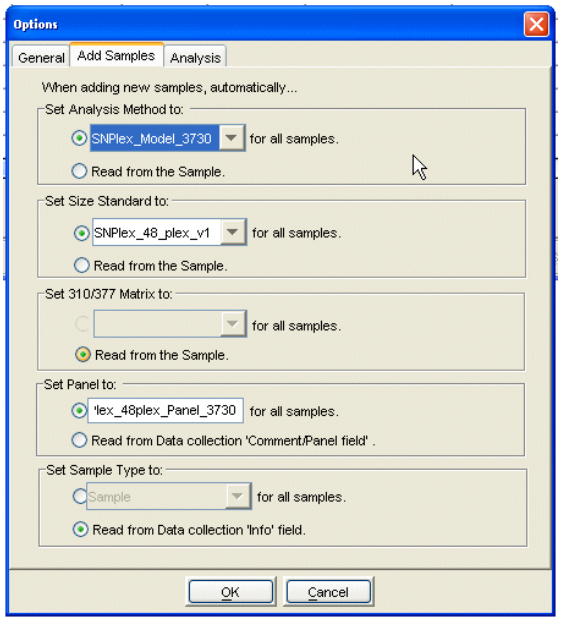
  IMPORTANT! For GeneMapper software to read SNP Set names from the plate record, you must import the AIF into GeneMapper software before importing the SNPlex System sample files (*.fsa).

- Depending on the way you set up your plate record (see "Creating Results Groups and Plate Records" on page 3-26), Analysis Method, Size Standard, and Panel information can be completed:
  - In the plate record before electrophoresis, using Data Collection software, typically by importing a formatted text file containing this information.
  - Automatically upon sample file import. (See "Setting Analysis Method, Size Standard, and Panel Automatically" on page 4-7.)
  - Manually, using GeneMapper software to edit the plate record

  Note: Applied Biosystems recommends using either of the first two methods. Manually entering the information by editing the plate record in GeneMapper software can be time-consuming and error-prone.

Setting Analysis Method, Size Standard, and Panel Automatically

To set Analysis Method, Size Standard, and Panel automatically:

1.	In the GeneMapper main window, select **Tools > Options**, then select the **Add Samples** tab.
2.	Specify the Analysis Method, Size Standard, and Panel as shown in the following figure.  The size standard and panel should be the same for all analyses. However, you can set the analysis method to either SNPlex_Model_3730 or SNPlex_Rules_3730, depending on which method you are using to analyze your samples. For more information about analysis methods, refer to **Appendix B**.
3.	Make sure that you set: • 310/377 Matrix to **Read from the Sample** • Sample Type to **Read from Data collection 'info' field**

4

Importing Sample Files

GeneMapper software retains the folder structure of imported sample files. That is, if the Data Collection software stores each run in a separate folder, GeneMapper software also creates one folder for each run.

For GeneMapper software to correctly analyze data, all sample files from a run must be stored in the same run folder. Additionally, an allelic ladder sample from the same run must also be included in each run folder. For 384-well plates, sample files should be stored in four run folders for 96-capillary arrays or eight run folders for 48-capillary arrays. The folder structure is generated by correctly setting up a Results Group, as described in "Creating Results Groups and Plate Records" on page 3-26.

To import sample files into GeneMapper software:

1.	Select **File > Add samples to project**.
2.	Find the data that you want to analyze. a. In the tree pane (right side of the workspace), click a folder to select it. b. Click **Add to list** to add the files contained in the folder. The files should appear in the list of files (left side of the workspace).
3.	After adding all relevant files, click **Add** to add the files to the project.

Analyzing SNPlex System Data

GeneMapper software v3.7 provides two methods for analyzing SNPlex System data, based on the clustering algorithms used to calculate the SNP quality. For more information about analysis methods, refer to **Appendix B**.

To analyze SNPlex System data:

1.	Before proceeding with analysis, check to see that: • Samples have the correct sample type designations – Allelic ladder samples are labeled as "allelic ladder". – No-template control samples are labeled as "negative controls". – All other samples are labeled "sample". • Analysis Method is set to either **SNPlex_Model_3730** or **SNPlex_Rules_3730** for all samples • Panel is set to **SNPlex_48plex_Panel_3730** for all samples • Size Standard is set to **SNPlex_48plex_v1** for all samples • SNP Set is set to the appropriate SNP set for each sample
2.	Click  **(Analysis > Analyze Samples)**.

Reviewing Results

Use the following guidelines for reviewing your data:

- **Review the bin offsets** — If the run was good, offsets should be small (<0.5). If offsets are large in one run but not in others, inspect the failed run.

  To view bin offsets for a run, select the run folder, then click **View** > **Bin Offsets**.

  Note: The software calculates bin offsets by comparing sample data to the allellic ladder for that run and assumes that all sample files within a folder are from the same run. For this reason, it is crucial that you keep samples from a run in the same folder.

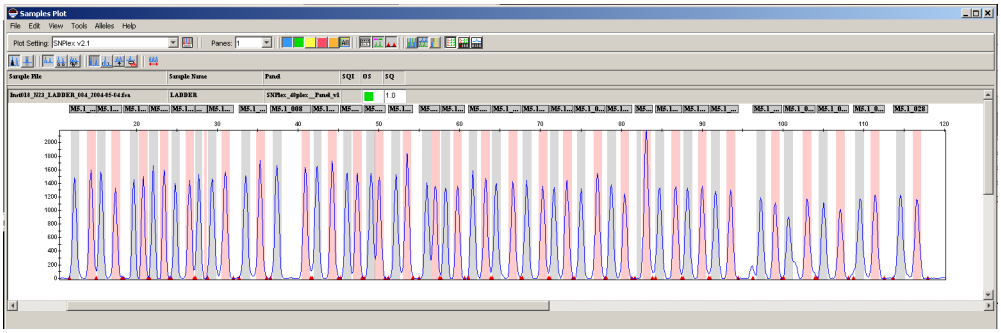
  For more information about sizing quality, refer to "Troubleshooting Sizing Quality" on page 5-10.
- **Review the sizing quality (SQ)** — Any sample that fails sizing is not used in the analysis.

  Select **Analysis** > **Size Map Editor**, then observe if the SQ values for size standards passed.

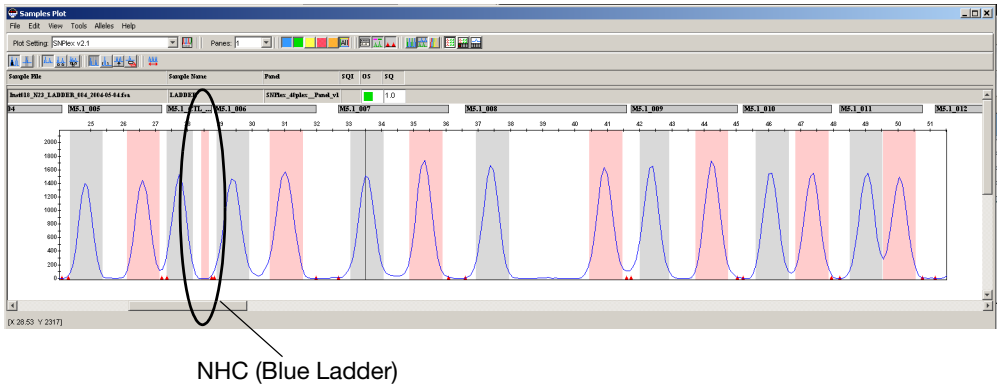
  For more information about sizing quality, refer to "Troubleshooting Sizing Quality" on page 5-10.
- **Review the allelic ladders** — Allelic ladder samples that do not pass the well quality (WQ) parameter are not included in the analysis. Check binning even for allelic ladder samples with passing WQs.

  Select **Analysis** > **Display Plots**, then observe if:

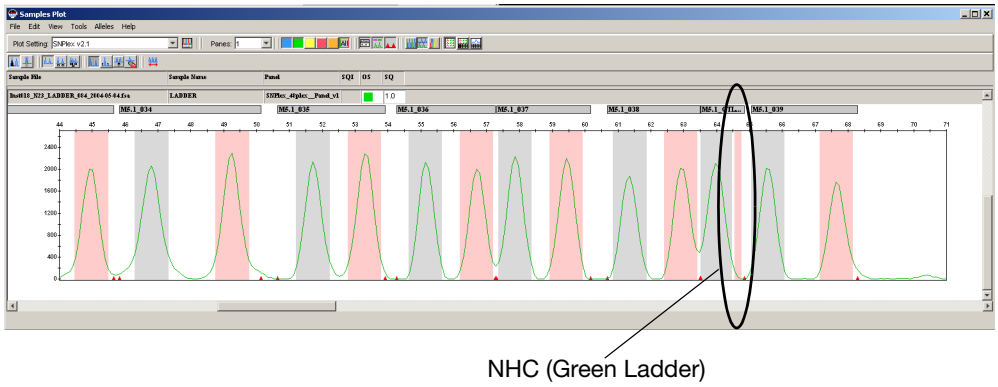
  – Each bin contains a single allelic ladder peak.



  – Each bin contains a single peak, except for the following bins, which should be empty:
    - Blue ladder: gray bin for M5.1_CTL_002_PHC
    - Blue ladder: pink bin for M5.1_CTL_001_NHC



- Green ladder: gray bin for M5.1_CTL_004_PHC
- Green ladder: pink bin for M5.1_CTL_003_NHC



For more information about allelic ladders, see "Troubleshooting Allelic Ladders" on page 5-18.

- **Review the cluster plots.**

  Select **Analysis** > **Display Cluster Plots**, then observe if:

  – Clusters are tight.

  – Signal strength is consistent.

  – There are any calls that you want to edit.

  For more information about cluster plots, see "Troubleshooting Cluster Plots" on page 5-26.

Exporting SNPlex System Data

To export:

- Genotype information – Select the **Genotype** tab, then select **File > Export Table**.
- Sample information – Select the **Sample** tab, then select **File > Export Table**.
- Both genotype and sample information – Select **File > Export Combined Table**.
- SNP Table – Once the cluster plot has been selected, select **File** > **Export Table**.

  Note: When exporting both types of information, you can select **File >Export Combined Table** regardless of which tab you are viewing.

You can also use the Report Manager feature of GeneMapper Software v3.7 to generate multi-column, custom reports from the data in the sample and genotype tables.

For more information about exporting SNPlex System data, refer to the GeneMapper software online help.

Troubleshooting 5

This chapter covers:

Troubleshooting Process

An effective way to carry out SNPlex™ system troubleshooting is to follow the three-step sequential process, as explained in the following table.

Step		Potential Problems	See Page
1	Inspect raw electrophoresis data	Problems arising from capillary electrophoresis, such as resolution or signal, can be identified by studying raw electrophoresis data. These problems may not be SNPlex System assay-related and can cause ambiguous results even when the chemistry is working properly.	5-3
2	Review GeneMapper® software analysis	Small errors in sample sheet setup, file type assignment, size-standard peak assignment, or binning of allelic ladders can cause GeneMapper software analysis to fail or to produce ambiguous results.	5-10
3	Study analyzed results and assay controls	If problems do not seem to be caused by mistakes in electrophoresis or GeneMapper software setup, they may be caused by chemistry-related issues, such as DNA quality, liquid handling, or thermal cycler programming. Inspecting assay controls, cluster-plot patterns, and electropherograms can help pinpoint the cause.	5-22

IMPORTANT! By performing these steps in the suggested sequence, you systematically rule out potential causes of a problem.

This chapter presents tables that guide you through the troubleshooting process when using 3730/3730*xl* instruments. Additionally, this chapter includes explanations of the potential problems cited in the flowcharts.

Some commonly used terms and their synonyms are:

- Signal strength – peak height
- Allelic ladder – SNPlex™ System ZipChute™ Mix

Troubleshooting Raw Data

The first step in the troubleshooting process is to rule out nonchemistry-related problems that may be caused by the capillary electrophoresis process. Viewing electrophoresis data allows you to identify problems that can lead to ambiguous results.

Use Figure 5-1 to troubleshoot problems related to the electrophoresis process.

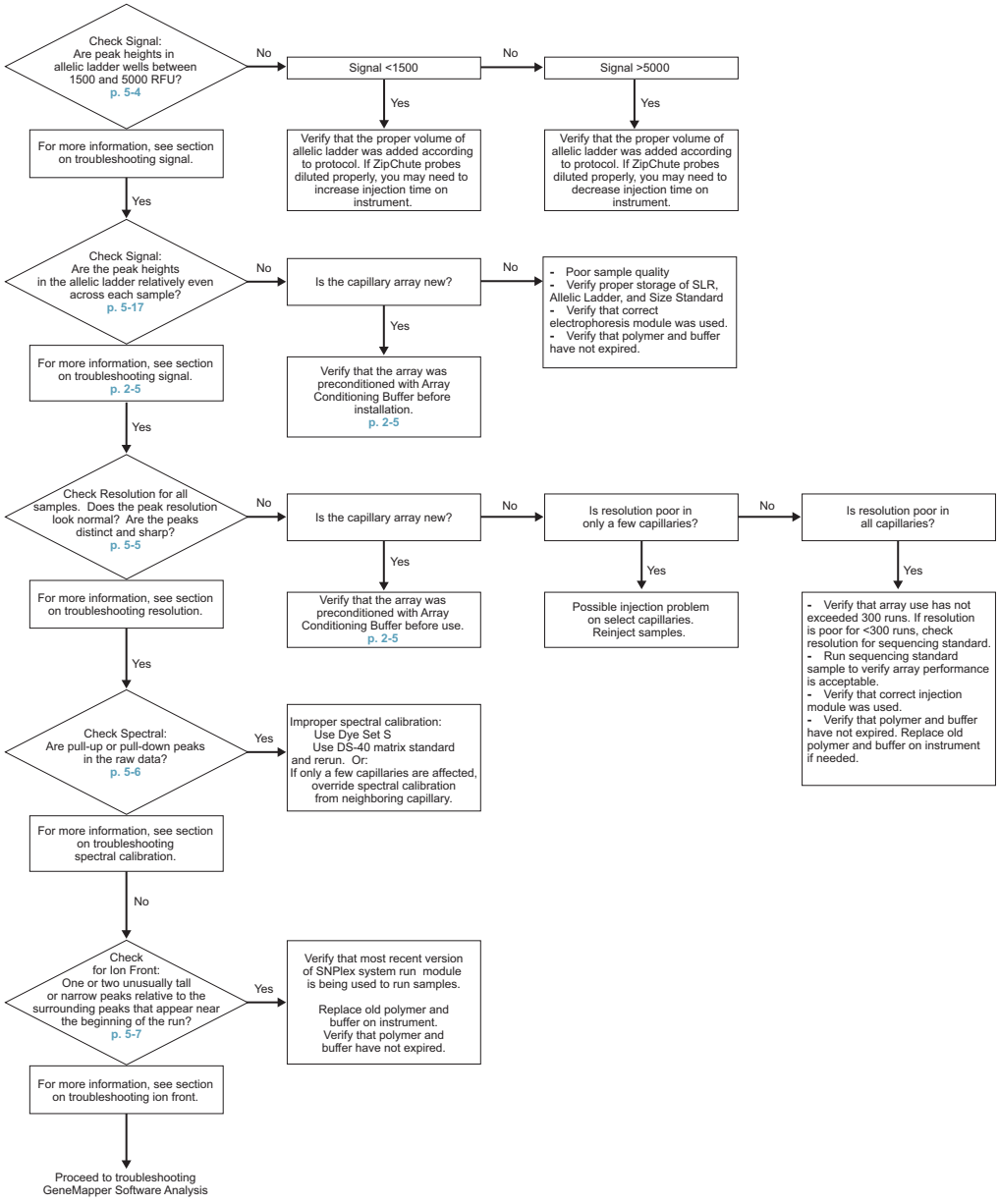


Figure 5-1 Workflow for troubleshooting raw data

Viewing Raw Data

- To view raw data using GeneMapper software, select **View > Raw Data**.
- To return to the samples view, select **View > Samples**.

Electrophoresis-Related Problems

Some problems that you can identify in the raw data:

- Signal strength of the allelic ladder not within the recommended 1500 to 5000 RFU range – See page 5-4.
- Signal stengths of the allelic ladder are uneven – See page 5-6.
- Poor resolution – See page 5-6.
- Pull-up or pull-down peaks (spectral calibration problems) – See page 5-6.
- A single peak or pair of peaks that is taller and narrower than adjacent peaks (ion front) – See page 5-7.

Examples of each problem and possible solutions are provided in subsequent sections of this chapter.

Troubleshooting Signal Strength

Example

Typical signal strengths for ZipChute™ probes in the allelic ladder are between 1500 and 5000 RFUs, as shown in Figure 5-2. Signals that are slightly outside of this range may still be considered normal. Also, signal strength can vary between instrument types and between instruments of the same type.

Although signal strengths significantly outside 1500 to 5000 RFU range do not necessarily mean that samples will fail, you should consider signal strength when adjusting run conditions for future runs. However, if signals for the allelic ladder are offscale, you must rerun samples.

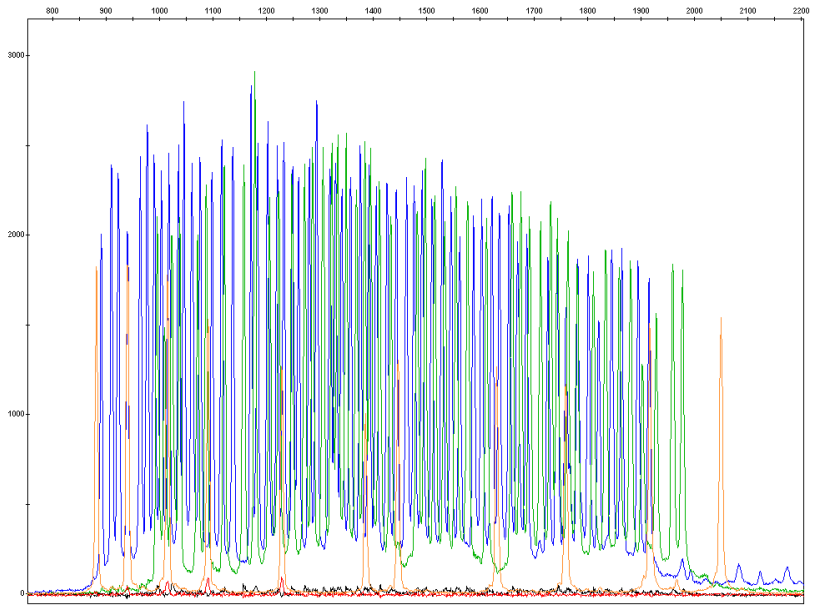


Figure 5-2 Raw data view of an allelic ladder with good signal strength

Solution

If the signal strength is significantly outside the expected range:

1. Confirm that the Allelic Ladder is appropriately diluted (see "Preparing Samples for Electrophoresis" on page 3-24).

2. If the allelic ladder is appropriately diluted, adjust the injection time to compensate for instrument-to-instrument variation.

   Note: The default injection time for the SNPlex System module is set to 10 s at 1 kV. Increasing the injection time increases the signal strength; decreasing the injection time decreases the signal strength.

   Note: Applied Biosystems recommends that you keep injection times between 5 and 20 seconds. Injection times outside this range result in poor resolution.

   a. Start the Data Collection software for the Applied Biosystems 3730 DNA Analyzer.
   b. Open the Module Manager, then click **New**.
   c. In the Run Module Editor, for:
      - Name – Enter a name for the module.
      - Type – Select **Regular**.
      - Template – For Data Collection v2.0, select **HTSNP_POP7_V2**,
        For Data Collection v3.0, select **HTSNP_POP7_V3**.
      - Injection Time – Enter a new injection time between 5 and 20 seconds.
   d. Click **OK** to save the module.

e. Open the Protocol Manager.

f. Create a new protocol using the module you just created. Alternatively, edit an existing protocol by replacing the currently selected module with the module you just created.

Troubleshooting Resolution

Example

For optimal results when running SNPlex System assay chemistry, precondition capillary arrays with Array Conditioning Buffer. Otherwise, the resulting data has poor resolution and inconsistent signal strength. Figure 5-3 poor resolution for a non-conditioned capillary.

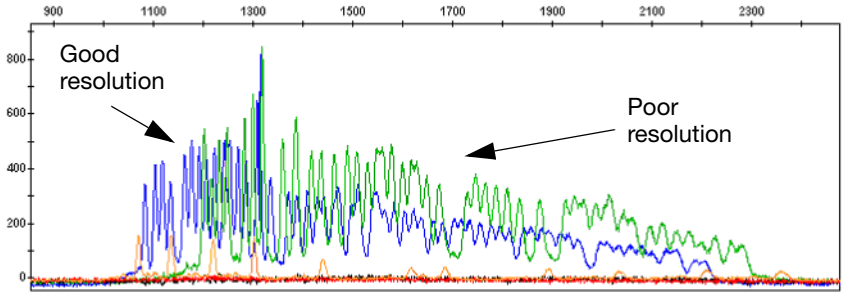


Figure 5-3 Raw data view of an allelic ladder showing poor resolution and loss of signal

Solution

If your raw data displays poor resolution:

- Ensure that all reagents in the instrument are fresh and that regular maintenance has been performed. Refer to the *Applied Biosystems 3730/3730xl DNA Analyzer Getting Started Guide* (PN 4359476).
- Precondition arrays before filling them with polymer or running samples. Refer to "Preconditioning the Capillary Array" on page 2-5.
- In cases where only one of several runs that were processed simultaneously shows poor resolution, try reinjecting samples a second time.

Troubleshooting Spectral Calibration

Example

Pull-up or pull-down peaks in raw data indicate problems with spectral calibration (Figure 5-4).

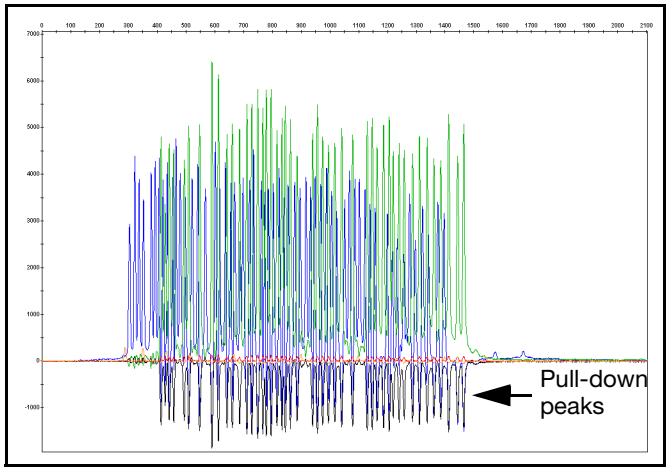


Figure 5-4 Raw data view of allelic ladder showing pull-down peaks

Solution

To prevent spectral calibration problems from occurring in future runs:

1. Inspect the raw data from each capillary for pull-up or pull-down peaks.
2. If you see pull-up or pull-down peaks from:
   - **Several capillaries** – Rerun the spectral calibration using Dye Set S.
   - **Only a few capillaries** – You can override the spectra in those capillaries with spectra from neighboring capillaries.

To override the spectra:

1.	Start the Data Collection software for the Applied Biosystems 3730 DNA analyzer.
2.	In the plate schematic, click the well(s) that you want to override, then click **Override Spectral**.
3.	From the drop-down list, select the nearest capillary with an acceptable spectral, then click **OK**.
4.	Click **Save**, then rename the calibration file. The Data Collection software uses the newly created spectral calibration file as the default.

Refer to the *Applied Biosystems 3730/3730xl DNA Analyzer Getting Started Guide* for more information about running and troubleshooting spectral calibrations.

Troubleshooting Ion Fronts

Example

An ion front typically affects a single peak or pair of peaks resulting in narrower and taller peaks than the adjacent peaks in the allelic ladder, as illustrated in Figure 5-5.

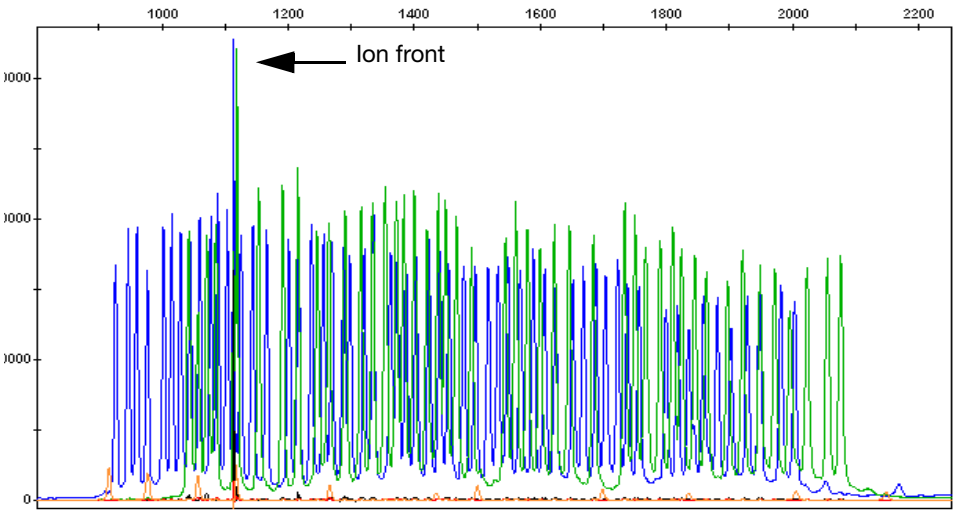


Figure 5-5 Raw data of allelic ladder showing a tall narrow peak resulting from an ion front

Ion fronts typically occur because of polymer aging in the instrument or the use of expired polymer lots.

The affected peak(s) can cause binning problems for an entire run because the ZipChute probe peaks in the front region do not migrate as expected by the software.

Solution

If you observe an ion front in your raw data:

- Verify that the Data Collection software is using run module HTSNP_POP7_V2 or later. This module incorporates a short prerun that generally eliminates the ion front.
- Replace the polymer and buffer on the instrument. Do not use old or expired lots of polymer and buffer.
- If you cannot rerun the samples with the correct module or with fresh buffer and polymer, or if the ion front persists after the rerun, you can analyze the data in GeneMapper software using the alternative analysis method described in the following section.

  Note: Although the settings in the alternative analysis method can help minimize the effects of ion fronts in some cases, you should not use these settings for regular analyses. When possible, replace the polymer and buffer and use an appropriate module.

To create an alternative analysis method to minimize ion fronts:

1.	Start the GeneMapper software (v3.7 or higher) .
2.	Select **Tools > GeneMapper Manager**, then select the **Analysis Methods** tab.

To create an alternative analysis method to minimize ion fronts: ***(continued)***

3.	Create a new analysis method based on the current method. a. From the list of analysis methods, select the current analysis method that you are using to analyze SNPlex System data. b. Click **Save As**. c. Enter a name for the new method. d. Click **OK**. The new method appears on the list of analysis methods. e. Click **Done**.
4.	Select the new analysis method, then click **Open**.
5.	In the Analysis Method Editor, select the **Peak Detector** tab.
6.	Change the Polynomial Degree to **7**. (Default is 5.)
7.	Change the Peak Window Size to **9**. (Default is 11.)
8.	Click **OK** to save the analysis method.
9.	Click **Done** to close the GeneMapper Manager, then reanalyze the samples using the new analysis method. Inspect the allelic ladder samples for correct binning.

Troubleshooting GeneMapper Software Analysis

The second step in the troubleshooting process is to rule out nonchemistry-related problems that may be caused by incorrectly setting up GeneMapper® software. Errors in sample sheet setup, file type assignment, size standard peak assignment, or allelic ladder binning can cause analysis to fail unless they are corrected.

Use Figure 5-6 to troubleshoot problems related to the software set-up process.

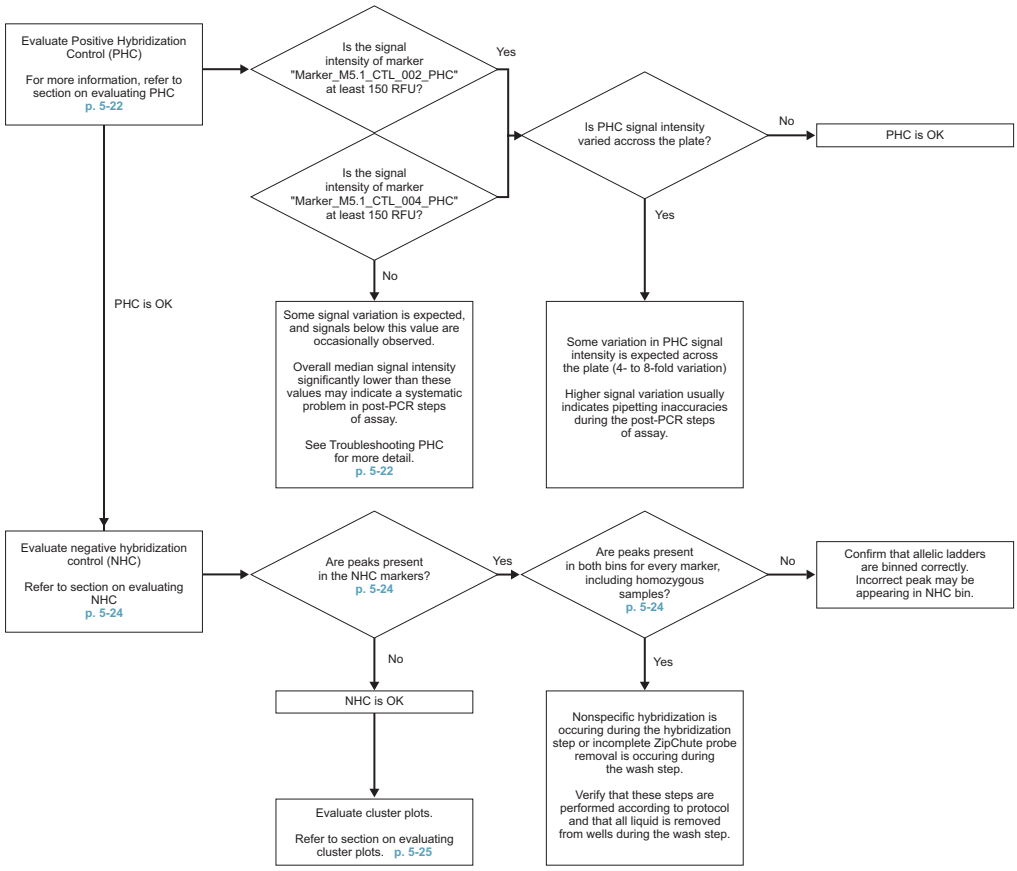


Figure 5-6 Workflow for troubleshooting GeneMapper software analysis

GeneMapper Software Setup Problems

Some problems that can arise from incorrectly setting up GeneMapper software are:

- Poor sizing quality – See page 5-10.
- Problems arising from allelic ladders – See page 5-18.

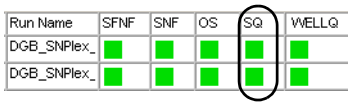
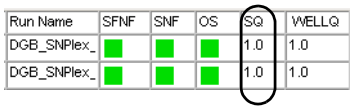
Examples of each problem, and possible solutions are provided in subsequent sections of this chapter.

Troubleshooting Sizing Quality

Determining Sizing Quality

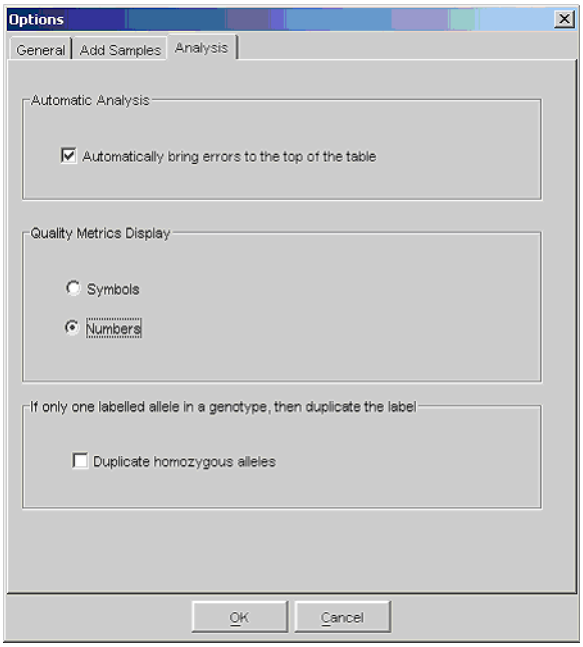
GeneMapper software sizing quality for SNPlex System results is indicated by symbols or numbers in the SQ (sizing quality) column of the Samples table. SQ values should be 0.98 or greater. Lower sizing quality values result in unreliable size calls.

By default, GeneMapper software displays colored symbols in the SQ column. However, when troubleshooting sizing quality, it is easier to use the numerical representation of sizing quality.

Sizing Quality Representation	Example Samples Table
Symbols (default)	
Numbers (recommended for troubleshooting)	

5

To switch sizing quality views in GeneMapper software:

1.	Select **Tools > Options**, then select the **Analysis** tab.
2.	Under Quality Metrics Display, select **Numbers**. 
3.	Click **OK** to apply your settings.

Determining Sizing Quality of Size Standards

If the sizing quality of a sample is less than 0.98, check the size standard for the sample using the Size Match Editor (select **Analysis > Size Match Editor**). For size standards to produce good sizing quality, all standard peaks should:

- Have signal strength >50 RFU.
- Have relatively uniform signal strengths.
- Be correctly identified by GeneMapper software.

Example: Good Sizing Quality

Figure 5-7 shows a size standard with good sizing quality. Note the even peak height and low background.

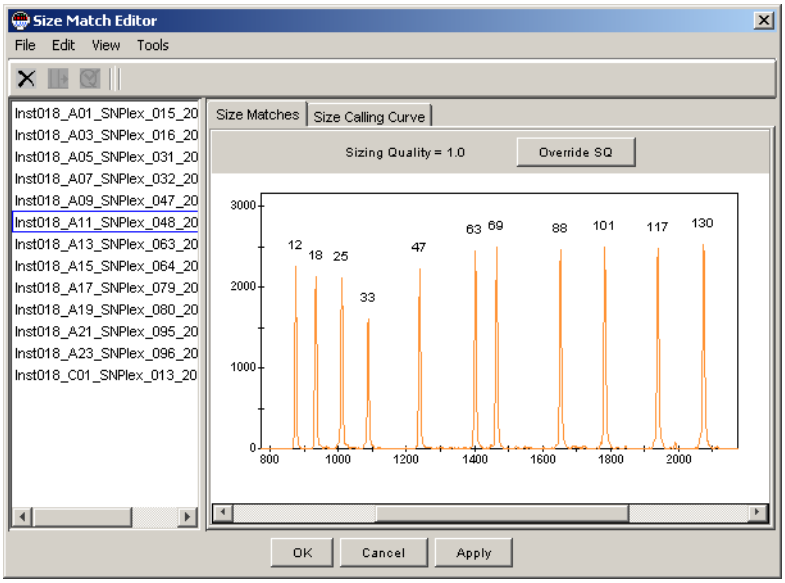


Figure 5-7 Size standard with good sizing quality

Example: Poor Sizing Quality

Figure 5-8 shows a size standard with poor sizing quality. Note the low signal in the figure below.

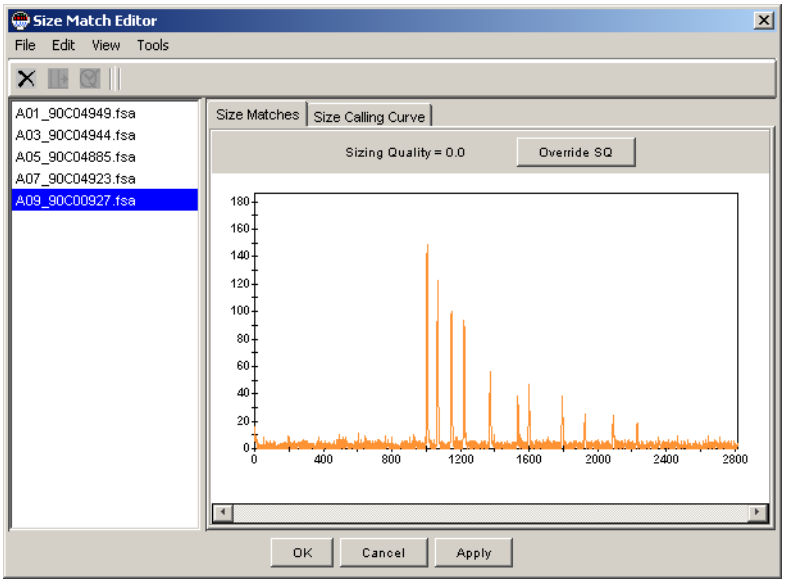


Figure 5-8 Size standard with poor sizing quality

5

Example: Size Standard Error

Figure 5-9 shows a size standard that failed due to no signal, which occurs when size standard is omitted from the sample or when the sample is not properly injected. Note that all signal strengths are below 12 RFU, compared to samples with good sizing quality (Figure 5-7), which have signal strengths above 200 RFU.

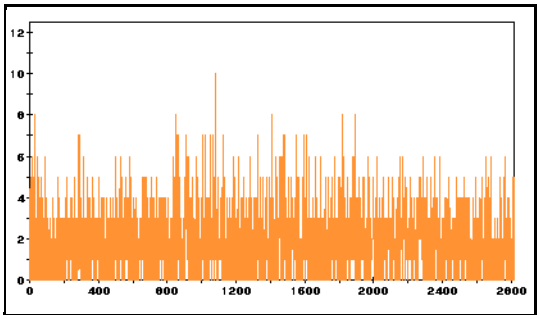


Figure 5-9 Size standard omitted from sample or not properly injected

Solution 1: Checking the Signal Strength

Use the Size Match Editor (**Analysis > Size Match Editor**) to make sure that all size-standard peaks have a signal of at least 50 RFU.

The default analysis method for analyzing SNPlex System data uses an orange threshold setting of 50. GeneMapper software does not identify any peaks that fall below this threshold, and consequently, sizing fails.

- Consistently low size-standard peaks across a single run may indicate incorrect dilution of size standard in sample loading reagent or incorrect injection settings.
- Low signal strengths for size standards in a small number of capillaries in a single run may result from electrophoresis or pipetting errors.
- If size calling appears accurate for most capillaries but inaccurate for others, the problem may be due to electrophoresis or pipetting errors. If the problem occurs in the same capillary over multiple runs when different samples are used, the capillary may be defective. If a capillary consistently fails for the same sample, the problem may be with the sample. Try placing the sample on a different position in the plate to rule out a capillary problem.
- If peaks are clearly distinguishable but fall below the orange threshold, you can try to adjust the analysis method so that the orange threshold value is less than that of the shortest peak.

To lower the orange threshold in the SNPlex System analysis method:

1.	Start the GeneMapper software.
2.	Select **Tools > GeneMapper Manager**, then select the **Analysis Methods** tab.

To lower the orange threshold in the SNPlex System analysis method: *(continued)*

3.	Create a new analysis method based on the current method. a. Select the current analysis method that you are using to analyze SNPlex System data. b. Click **Save As**. c. Enter a name for the new method. d. Click **OK**. The new method appears in the list of analysis methods. e. Click **Done**.
4.	Select the new analysis method, then click **Open**.
5.	In the Analysis Method Editor, select the **Peak Detector** tab.
6.	Change the Peak Amplitude Threshold for orange to a value less than the signal intensity of the shortest size-standard peak. Do *not* alter other color settings. **Note:** Applied Biosystems recommends that you keep the orange threshold value at 50.
7.	Click **OK** to save the analysis method.
8.	Click **Done** to close the GeneMapper Manager, then reanalyze the samples using the new analysis method.

Solution 2: Verifying GeneMapper Software Size Calls

Use the Size Match Editor to make sure that all size-standard peaks are identified correctly by GeneMapper software.

When the signals of the size-standard peaks are very high, GeneMapper software may incorrectly identify a small shoulder preceding a peak as the main peak (shown in Figure 5-10). Because this shoulder peak does not occur at the correct position relative to the other peaks, sizing fails.

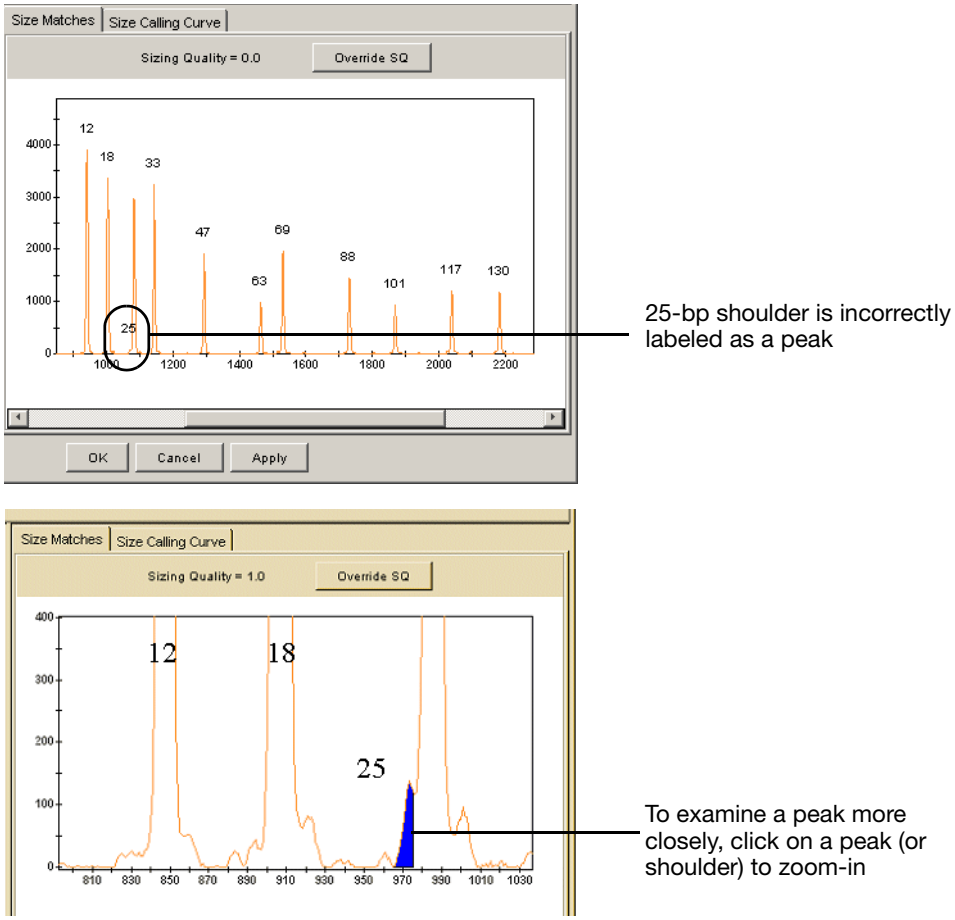


Figure 5-10 Size standard with shoulder incorrectly labeled as a peak

By adjusting the analysis method so that the orange threshold value is greater than the height of the shoulder, you may achieve good sizing.

To raise the orange threshold in the SNPlex System analysis method:

1.	Start the GeneMapper software.
2.	Select **Tools > GeneMapper Manager**, then select the **Analysis Methods** tab.

To raise the orange threshold in the SNPlex System analysis method: *(continued)*

3.	Create a new analysis method based on the current method. a. Select the current analysis method that you are using to analyze SNPlex System data. b. Click **Save As**. c. Enter a name for the new method. d. Click **OK**. The new method appears in the list of analysis methods. e. Click **Done**.
4.	Select the new analysis method, then click **Open**.
5.	In the Analysis Method Editor, select the Peak Detector tab.
6.	Change the Peak Amplitude Threshold for orange to a value greater than the signal intensity of the shoulder peak.
7.	Click **OK** to save the analysis method.
8.	Click **Done** to close the GeneMapper Manager, then reanalyze the samples using the new analysis method.

Solution 3: Checking Evenness of Signal Strength

Use the Size Match Editor (**Analysis > Size Match Editor**) to make sure that all size-standard peaks have reasonably even signal strengths.

To rule out instrument-related problems, run a plate of ZipChute™ ladder with size standard, as described in "Preconditioning the Capillary Array" on page 2-5.

Troubleshooting Allelic Ladders

GeneMapper software determines the positions of allelic ladder peaks for each run, then compensates for offset between the positions of the bins and those of the ladder peaks. Through this process, the software compensates for run-to-run variations that can occur in electrophoresis and ensures that experimental peaks are assigned to bins correctly.

Example

Figure 5-11 shows an allelic ladder where all peaks have a relatively even signal and are binned correctly.

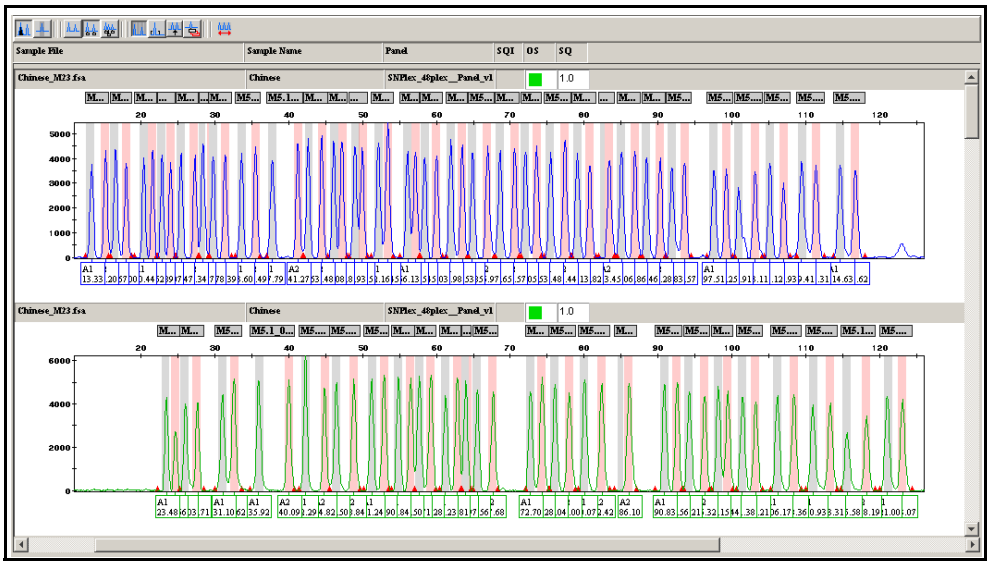


Figure 5-11 Good allelic ladder sample with even signal and accurate binning

Viewing Allelic Ladders in Plot View

To inspect the allelic ladders for a run, open them in Plot View (**Analysis > Display Plots**), then select the SNPlex System plot settings from the Plot Setting menu.

As shown in Figure 5-11, the green and blue SNPlex System allelic ladders should be displayed independently, and bins should be superimposed over the ladder peaks.

Note: If peaks are not within bins, establish proper binning before any further analysis.

Solution 1: Confirming That Allelic Ladders Are Injected With Each Set of Samples

Because the GeneMapper software uses information from allelic ladders to compensate for run-to-run variations, allelic ladders need to be included with each injection of samples to calculate and apply the necessary bin offset to all samples.

If an allelic ladder is not included or omitted from a run, the software attempts to use the default bin settings without any bin offsets when analyzing the samples. If the peak positions of a sample differ from those of the default bin settings, binning can fail. This failure can be corrected by running an allelic ladder and measuring the required offset.

Reinject samples that have been run without an allelic ladder using an allelic ladder.

Solution 2: Confirming File-Type Assignment

The GeneMapper software requires that allelic ladder wells be designated as samples of type “allelic ladder” in the “Sample Type” column. If allelic ladder wells are mistakenly designated as “sample” or “positive control,” the software does not use this allelic ladder when calculating bin offsets.

If necessary, assign the correct sample type to the allelic ladder, then reanalyze the samples.

To reassign sample types:

1.	Locate the allelic ladder sample(s) in the Samples View.
2.	For each ladder sample, verify that the sample type (indicated in the Sample Type column) is “Allelic Ladder.”
3.	If the sample is labeled incorrectly, click the sample type, then select **Allelic Ladder** from the popup menu.
4.	Reanalyze the sample.

Solution 3: Checking Accuracy of Peak Assignment

Occasionally, the GeneMapper software misidentifies a peak in the allelic ladder, causing problems with binning offsets. In the example in Figure 5-12, the software misidentified a 78-RFU peak as the first peak in the green ZipChute ladder. Consequently, the first four bins in the ladder are incorrectly positioned.

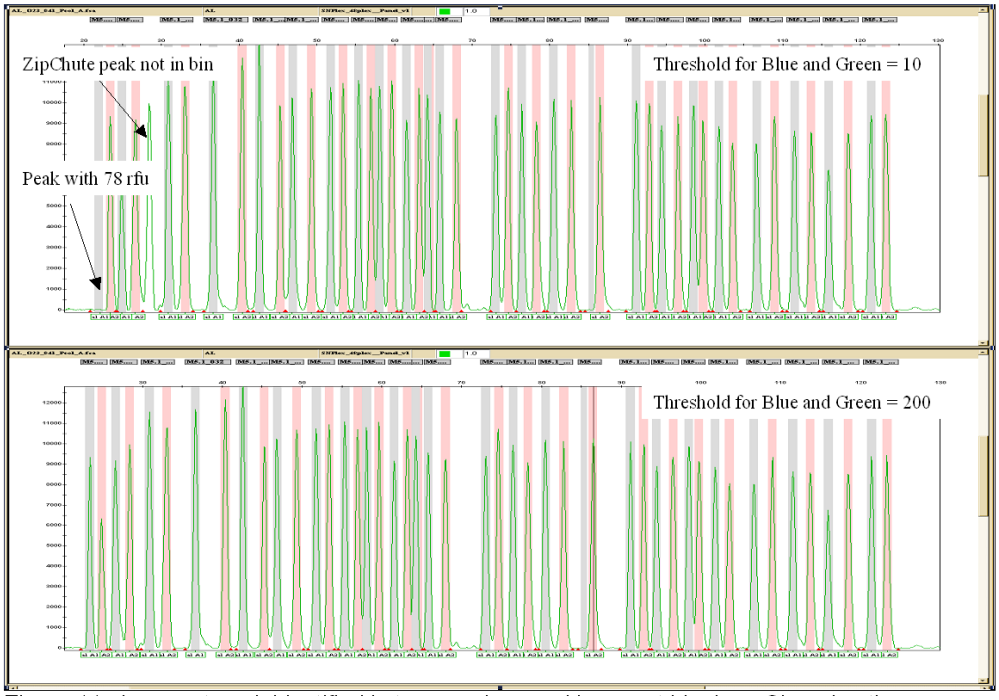


Figure 5-12 Top: Example with incorrect binning; Bottom: Example with correct binning

In this example, generating a separate analysis method for the allelic ladders can correct the problem. Adjust the threshold for the color of the misidentified peak (in this case, green) to a higher value to ignore the peak. Adjusting the green threshold to 200 in the analysis method causes the software to ignore the first peak (because it is less than 200 RFU) and corrects the problem.

Note that this type of problem can occur in either the green or the blue allelic ladder.

To adjust the blue or green threshold in the SNPlex System analysis method:

1.	Start the GeneMapper software.
2.	Select **Tools > GeneMapper Manager**, then select the **Analysis Methods** tab.

To adjust the blue or green threshold in the SNPlex System analysis method: ***(continued)***

3.	Create a new analysis method based on the current method. a. Select the current analysis method that you are using to analyze SNPlex System data. b. Click **Save As**. c. Enter a name for the new method. Select a name that easily identifies it as a special ladder analysis method. d. Click **OK**. The new method appears in the list of analysis methods. e. Click **Done**.
4.	Select the new analysis method, then click **Open**.
5.	In the Analysis Method Editor, select the **Peak Detector** tab.
6.	Change the Peak Amplitude Threshold for green or blue to a value greater than the signal intensity of the misidentified peak.
7.	Click **OK** to save the analysis method.
8.	Click **Done** to close the GeneMapper Manager, then reanalyze the samples using the new analysis method for only the allelic ladder wells.
9.	**IMPORTANT!** Use the standard analysis method to analyze the rest of the samples. **IMPORTANT!** Setting the blue or green thresholds higher than the default value (10 RFU) can cause the software to miss sample peaks. Use analysis methods with modified threshold settings only for analyzing allelic ladder samples; use the standard analysis method to analyze all other samples.

Solution 4: Removing an Allelic Ladder from a Project

GeneMapper software averages all allelic ladders from a run when determining binning offsets. A misbinned ladder can throw off the average for the entire run.

When you cannot correct problems in an allelic ladder, you may have to remove the ladder from the project to achieve correct binning.

To remove an allelic ladder, go to the Samples View, click the defective ladder, then select **Edit > Delete From Project**. Reanalyze the samples after removing the problematic ladder.

Note: Removing all ladders from a run can cause serious binning problems. Make sure to examine samples for correct binning.

Troubleshooting Analyzed Data

Problems that are not attributable to the electrophoresis or GeneMapper software set up processes may be related to the assay chemistry. Troubleshooting SNPlex System assay chemistry involves inspecting assay controls, cluster plot patterns, and electropherograms.

Use Figure 5-13 to troubleshoot problems related to SNPlex System assay chemistry.

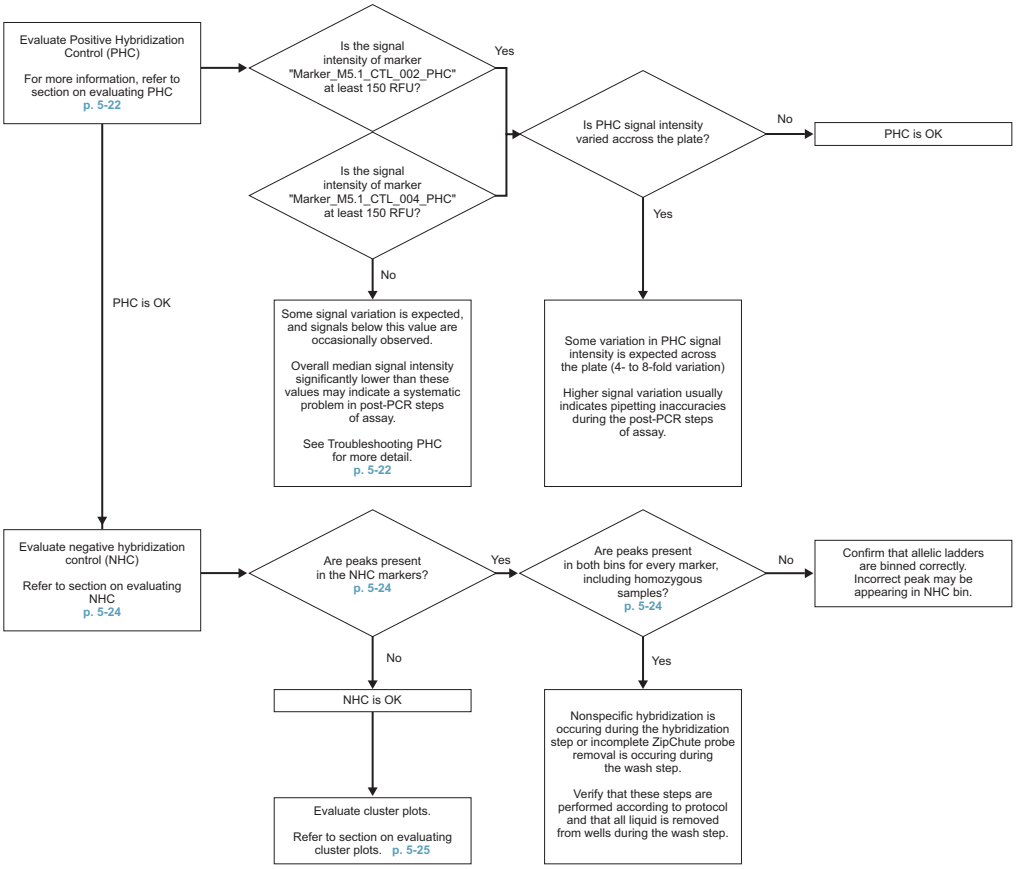


Figure 5-13 Workflow for troubleshooting analyzed data

Chemistry Problems

To troubleshoot problems that can arise from the assay chemistry, you can analyze information from:

- Positive hybridization controls – See page 5-22.
- Negative hybridization controls – See page 5-25.
- Cluster plots – See page 5-26.

Troubleshooting Positive Hybridization Controls

The Positive Hybridization Control (PHC), which is added to the binding buffer when PCR products are bound to streptavidin plates, is used to monitor the efficiency and reproducibility of the ZipChute probe hybridization and elution steps. It consists of two single-stranded biotinylated oligonucleotides containing a ZipChute probe binding site.

When analyzed in GeneMapper software, every well should include two PHC peaks, one for each marker (M5.1_CTL_002_PHC and M5.1_CTL_004_PHC). Both peaks should fall into the second bin (allele 2), and cluster plots for the PHC should display a single cluster associated with allele 2.

Example

Figure 5-14 shows PHC signals from a single 96-capillary run. Some variation in signal intensity within a run is acceptable. The signal intensity of the encircled sample is significantly lower than the rest of the samples, indicating potential problems with post-PCR assay steps.

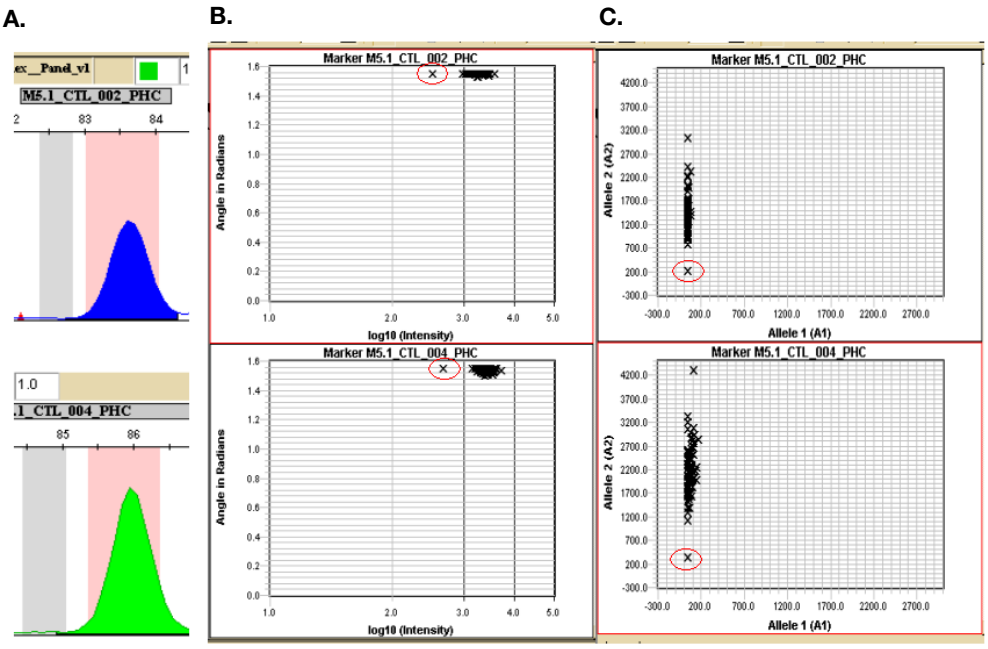


Figure 5-14 PHC signals from a single 96-capillary run. A: Electropherograms of the two PHC peaks; B: PHC signals from all samples in polar plot view; C: PHC signals from all samples in Cartesian plot view

Step 1: Evaluating Overall Median Signal Intensity

Variations in signal intensity between runs or instruments can be expected. The typical overall median signal intensity ranges from 150 – 2400 RFU.

Overall median signal intensities significantly lower than these values may indicate a problem in the post-PCR steps of the assay. For example, incorrect dilution of PHC in binding buffer, using binding buffer instead of ZipChute hybridization buffer during the ZipChute probe elution step, or incomplete removal of hybridization wash buffer before ZipChute probe elution can cause overall signal intensity to drop.

Step 2: Evaluating Variation in Signal Intensity Across the Plate

Variations in PHC signal intensity between runs or instruments can also be expected. Variations up to an eightfold difference are acceptable. Anything more indicates possible problems with post-PCR assay procedures, typically related to pipetting inaccuracies.

If you notice large variations in signal intensity across a plate, inspect and recalibrate pipettes and robots as necessary.

Troubleshooting Negative Hybridization Controls

The Negative Hybridization Control (NHC) is used to monitor nonspecific hybridization that may occur during the hybridization step. It consists of two ZipChute markers (M5.1_CTL_001_NHC and M5.1_CTL_003_NHC) whose binding sites are not complementary to the products generated during the SNPlex System assay.

Example

When analyzed in GeneMapper software, each well should include the two NHC markers. As shown in Figure 5-15, no peaks should be visible for either marker, and signal intensity of the markers should be less than 150 RFU. Higher RFU values may indicate a software binning problem or problems with the hybridization or washing steps of the assay.

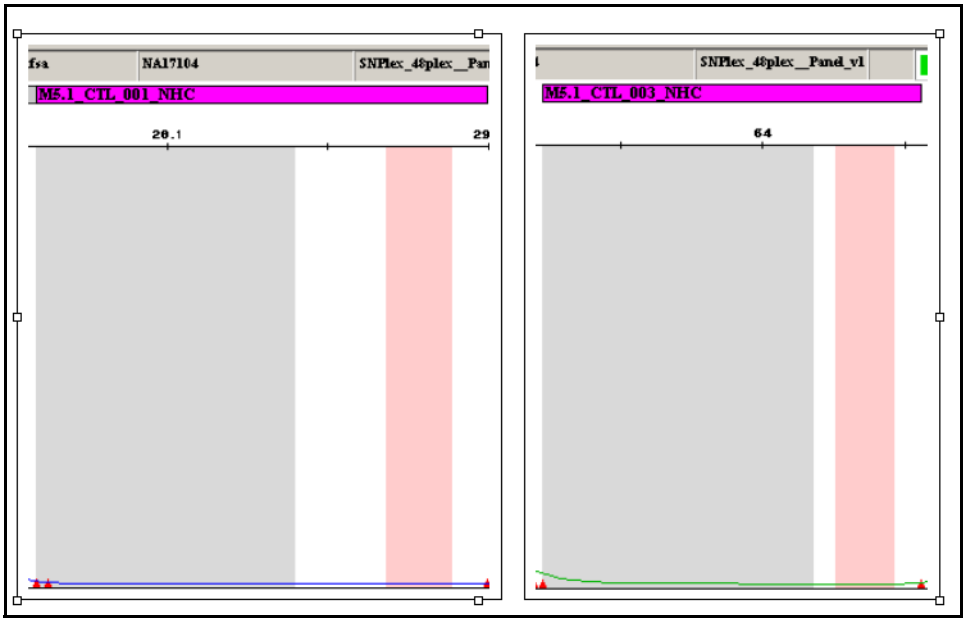


Figure 5-15 Electropherograms showing NHC markers

Observation: Peak Appears in NHC

Peaks in NHC markers are almost always caused by binning errors. If you observe peaks for NHC markers, review the allelic ladders to confirm that all peaks are correctly binned.

Observation: Peak Appears in NHC Even with Correct Binning

True peaks in NHC markers indicate problems with the hybridization or wash steps of the assay. When peaks appear in NHC markers, it is likely that the peaks appear in each bin for all markers, even for samples that are homozygous.

If you observe true peaks for NHC markers, confirm that you are performing both the hybridization and wash steps according to the protocol. Further, ensure that you perform the wash steps thoroughly, removing all the liquid from the plate before continuing with the next steps.

Troubleshooting Cluster Plots

Cluster plots, when used with other troubleshooting tools such as PHCs and NHCs, help you narrow down the causes of a problem. Unexpected patterns in cluster plots can originate from components of SNPlex System chemistry, DNA quality, pipetting and liquid handling, robotics, and capillary electrophoresis.

Example

Figure 5-16 shows cluster plots from successful SNPlex System assays.

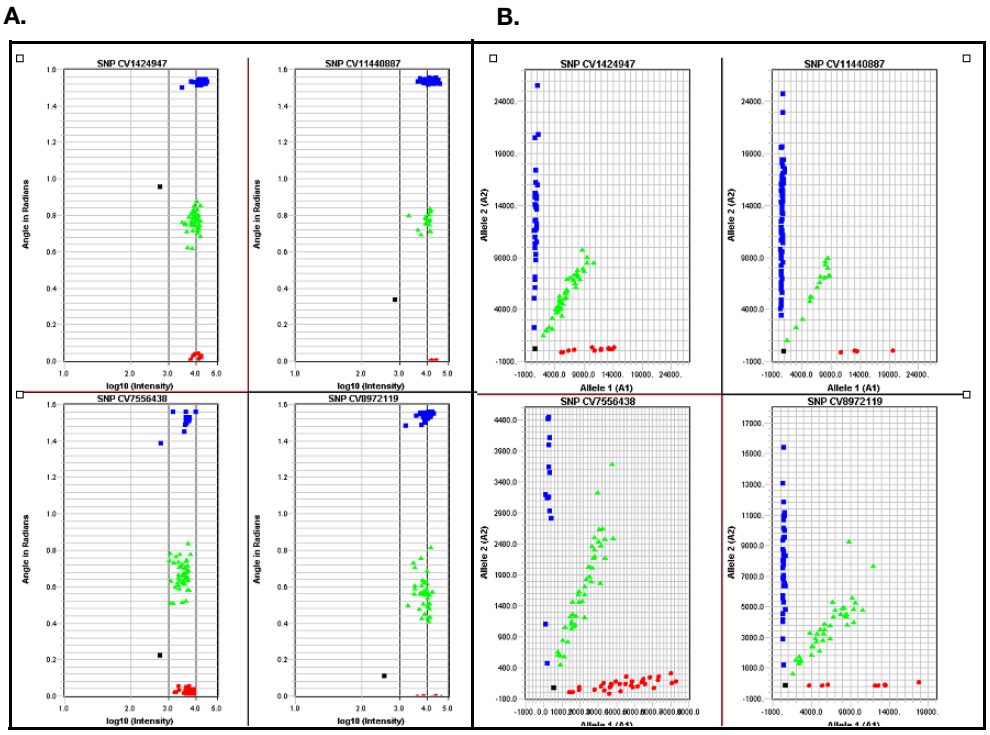


Figure 5-16 Cluster plots from successful SNPlex System assays. A: Polar view; B: Cartesian plot view

The following sections present examples of typical patterns observed in cluster plots. The table following each plot describes possible causes when evaluated with the PHC, NHC, and size standard.

Example: Data Points Have Low Signal Intensity and Little to No Cluster Formation

Figure 5-17 illustrates cluster plots in which:

- Most of the data points have $\log_{10}$ intensity <3
- There are no clearly defined clusters

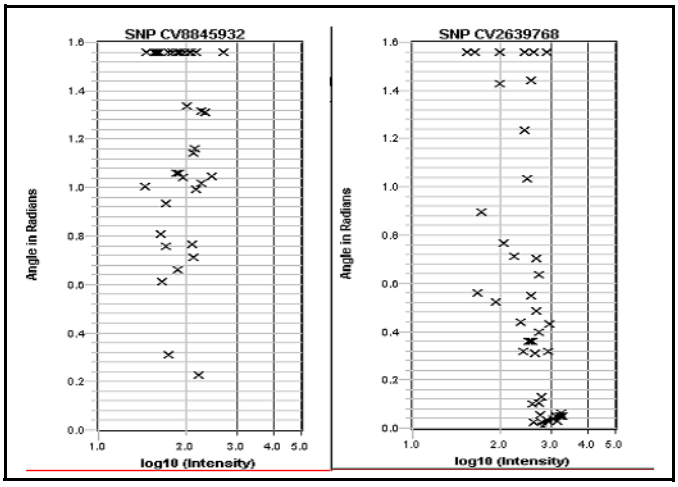


Figure 5-17 Cluster plots with low signal intensity and poor cluster information

Table 5-1 Troubleshooting cluster plots with low signal and poor cluster information

PHC	NHC	Size Standard	Potential Cause	Possible Solution
OK	OK	OK	Insufficient DNA	Confirm that input quality of DNA is adequate.
			Incomplete or excessive DNA fragmentation	Check distribution of DNA fragments on agarose gel.
			DNA contains OLA inhibitors	Repurify DNA.
			Used plate cover that is incompatible with the SNPlex System assay	Use recommended plate cover (see Table 1-3 on page 1-10)
			Concentration of OLA probe, ligase, or PCR primer is too low	Verify that quantities and concentrations of components used are as specified in the protocol.
			Incorrect OLA or PCR thermal cycling conditions	Ensure that thermal cycling conditions used are as specified in the protocol.
			Exonuclease step omitted	Repeat experiment with exonuclease step.
			Exonuclease mix kept at room temperature for more than 1 hour before use.	Prepare fresh exonuclease mix and repeat experiment.
			Insufficient PCR product transferred during post-PCR step	Check protocol. Evaluate and optimize pipetting accuracy in assay workflow.
			NaOH denaturation step omitted	Repeat experiment with denaturation step.

Table 5-1 Troubleshooting cluster plots with low signal and poor cluster information *(continued)*

PHC	NHC	Size Standard	Potential Cause	Possible Solution
Low signal	OK	OK	Low ZipChute mix concentration	Check concentration of ZipChute mix added to hybridization master mix.
			Wrong ZipChute dilution buffer used for hybridization master mix	Repeat experiment using the correct ZipChute dilution buffer.
			ZipChute dilution buffer not properly diluted	Repeat experiment using ZipChute dilution buffer at correct concentration.
			Bound ZipChute probes stripped off plate	Reconfigure the plate washer. Set the aspiration tip depth so that 15 to 20 µL of Wash Buffer remains in each well after each aspiration.
			Temperature too high during ZipChute probe hybridization	Check temperature in hybridization oven and correct, if necessary.
Low signal	OK	Low signal	Incorrect electrophoresis protocol—injection time too short	Confirm that correct electrophoresis protocol was used.
				Increase injection time.
				Run allelic ladder plate and reoptimize electrophoresis performance.
			High salt in sample	Remove all liquids from wells (by centrifuging plates ***upside down***) before adding sample loading mix.

Note: If the control DNA shows a signal that is significantly higher and outside one of the clusters you observe with your own DNAs, assume that the quality of your DNA is low and/or suboptimal concentrations of DNA were added [see "3730xl Instrument (96-capillary), 96-wells" on page 3-3,"3730xl Instrument (96-capillary), 384-wells"and "3730 Instrument (48-capillary), 96-wells" on page 3-4, "3730 Instrument (48-capillary), 384-wells" on page 3-5, and "Dispensing gDNA into Reaction Plates" on page 3-8].

Example: Tight Clustering of Data Points with Few Low-Signal Intensity Outliers

Figure 5-18 illustrates cluster plots in which:

- Most data points fall into tight, discernible clusters
- Several data points fall outside clusters (outliers)
- Clustered data points have signal intensity of $\log_{10} > 3$
- Outliers have signal intensity of $\log_{10} < 3$

In such cases, GeneMapper software is unable to identify clusters because of the presence of outliers. After the outliers are removed, the software correctly identifies the clusters.

This pattern suggests that the assay chemistry is working but that errors are occurring in specific samples.

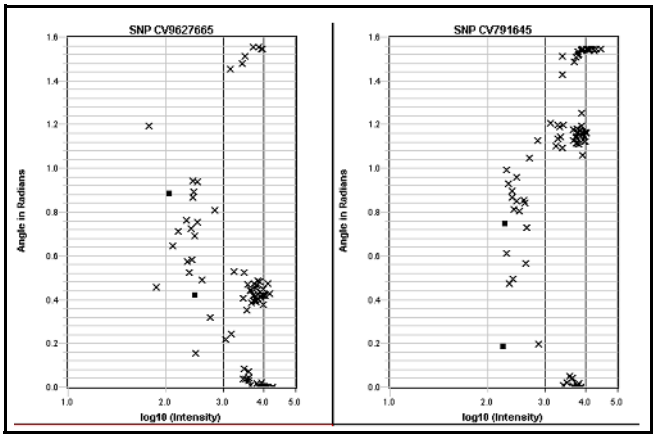


Figure 5-18 Cluster plots with discernible clusters and low-signal intensity outliers

Table 5-2 Troubleshooting cluster plots with discernible clusters and low-signal intensity outliers

PHC	NHC	Size Standard	Potential Cause	Possible Solution
OK	OK	OK	Insufficient DNA in wells with low signal	Confirm that input quantity of DNA is adequate.
			Incomplete or excessive DNA fragmentation	Check distribution of DNA fragments on agarose gel.
			DNA contains OLA inhibitors	Repurify DNA.
			Pipetting errors during OLA, PCR, exonuclease digestion, or PCR transfer for wells with low signal	Evaluate and optimize pipetting accuracy in assay workflow.

Table 5-2 Troubleshooting cluster plots with discernible clusters and low-signal intensity outliers

PHC	NHC	Size Standard	Potential Cause	Possible Solution
Low signal for outliers	OK	OK	Streptavidin failure in wells with outliers	Repeat experiment with new streptavidin plate.
			Pipetting errors in post-PCR steps	Evaluate and optimize pipetting accuracy in post-PCR assay workflow.
			Bound ZipChute probes stripped off plate	Reconfigure the plate washer. Set the aspiration tip depth so that 15 to 20 µL of Wash Buffer remains in each well after each aspiration.
Low signal for outliers	OK	Low signal for outliers	Capillary injection failure	Reinject samples.
				Run allelic ladder plate and optimize electrophoresis performance.
			High salt in sample	Remove all liquids from wells (by centrifuging plates ***upside down***) before adding sample loading mix.

Example: Tight Clustering of Data Points with Outliers at Different Angles

Figure 5-19 illustrates cluster plots in which:

- Most data points fall into tight, discernible clusters
- Several data points fall outside clusters (outliers)
- All data points (clustered and outlying) have signal intensity of $\log_{10} > 3$
- Outliers are present in different angles, as measured in the polar plot

The vertical axis in the polar plot represents the angle, in radians, between the x-axis and the data points in the Cartesian plot. Data points that extend at a different angle in the plot may indicate:

- Sample contamination
- Secondary mutation close to the SNP site on one allele for a subset of the DNA samples
- Allele duplication

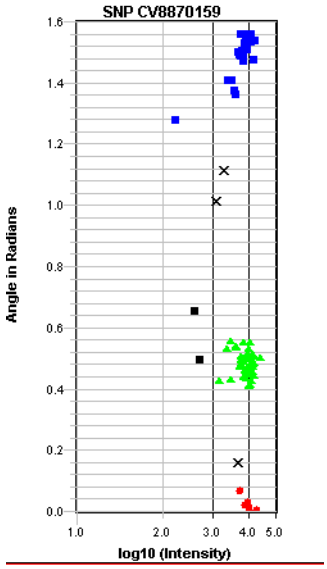


Figure 5-19 Polar plots with discernible clusters and outliers present at different angles

Table 5-3 Troubleshooting cluster plots with discernible clusters and outliers present at different angles

PHC	NHC	Size Standard	Potential Cause	Possible Solution
OK	OK	OK	DNA samples contaminated with DNA of a different genotype	Repeat experiment with uncontaminated DNA.
				Decontaminate pipettors or robotic tips with 10% bleach solution.
			OLA cross-contamination of some samples	Evaluate and optimize pipetting accuracy in assay workflow.
			Secondary SNP within genome-equivalent region of OLA probe sequence	Redesign probe set using alternative SNP.
			Allele duplication	

Example: Good Signal Intensity but Data Points Smeared Across Y-Axis (Angle); Poor Cluster Formation

Figure 5-20 illustrates cluster plots in which:

- All data points have signal intensity of $\log_{10} > 3$
- No discernible clusters are formed
- Samples appear to be smeared or stretched vertically in the polar plot

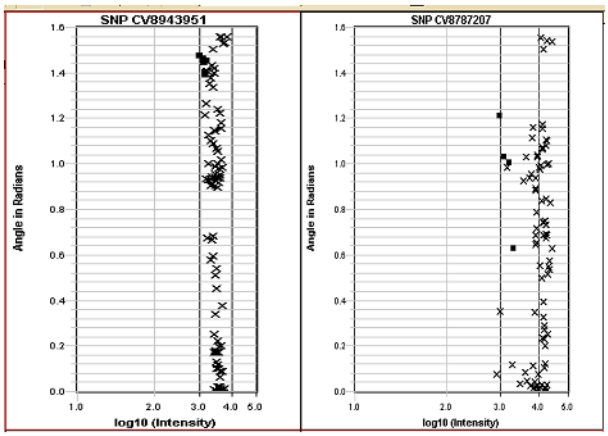


Figure 5-20 Cluster plots with good signal intensity but poor cluster formation

Table 5-4 Troubleshooting cluster plots with good signal intensity but poor cluster formation

PHC	NHC	Size Standard	Potential Cause	Possible Solution
OK	OK	OK	DNA samples contaminated with DNA of a different genotype	Repeat experiment with uncontaminated DNA.
				Decontaminate pipettors or robotic tips with 10% bleach solution.
			Off-scale peaks result in spectral pull-up in a bin of a different color, causing angle distortion in the SNP clusters. See Figure 5-21 for further explanation.	Too much PCR product transferred to post-PCR step. Adjust amount of PCR product transferred to streptavidin plates.
				Reduce injection time for capillary electrophoresis.
				Additional dilution of samples with sample loading reagent containing size standard.
			OLA probes are not specific to a unique locus—two different regions may be genotyped by one probe set **Note:** Strong possibility if nonhuman SNPs are assayed and no genome screen is available during probe design.	SNP may not be assayable.
			Used plate cover that is incompatible with the SNPlex System assay	Use recommended plate covers (see Table 1-3 on page 1-10)
High signal	High signal	OK	Insufficient washing after ZipChute probe hybridization	Reoptimize washing steps, ensuring that all liquid is removed from wells at each step.
High signal	High signal	High signal	Incorrect CE protocol – injection too long	Verify that correct electrophoresis protocol was used.
				Decrease injection time.
				Run allelic ladder plate and reoptimize electrophoresis performance.

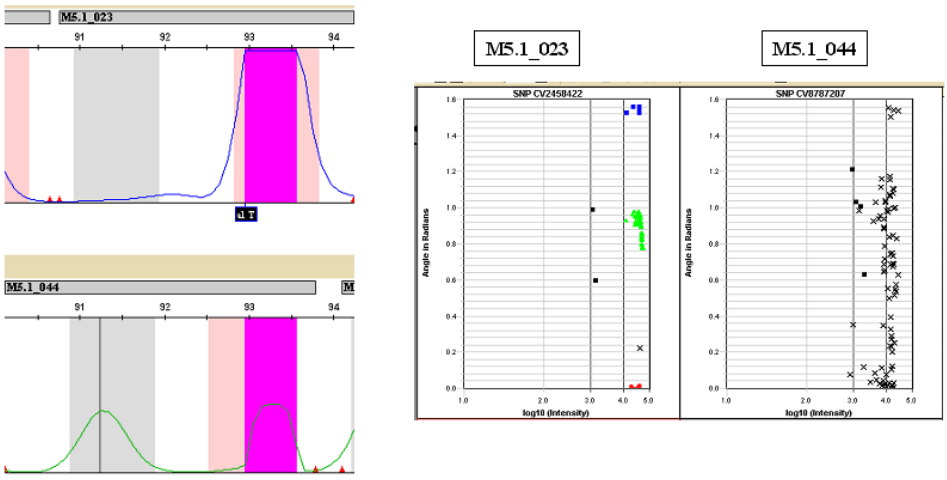


Figure 5-21 Off-scale peaks cause spectral pull-up

As illustrated in Figure 5-21, blue off-scale peaks in M5.1_023 cause a background signal due to the spectral pull-up in M5.1_044. As a result, angle differences occur during clustering for this marker, producing data points that are “smeared” along the Y-axis.

Using the SNPlex System Control Set

A

This chapter covers:

Product Description

The SNPlex™ System Control Pool (PN 4362635) is a set of ligation probes that can be used together with the SNPlex™ System Dried gDNA Plates Kit (PN 4362637) to evaluate the performance of the SNPlex System. When used with SNPlex™ System kits and reagents, the ligation probes in the control pool detect 48 human SNPs in a single mutliplex reaction.

About the Control Pool SNPs

The Control Pool SNPs

- Were selected from a list of SNPs for which validated TaqMan® assays were available
- Typically have a minor allele frequency of at least 0.1 in at least one of the following populations: African-American, Caucasian, Japanese, Chinese
- Were validated by individually genotyping 180 DNA samples selected from the four major populations with TaqMan® probe-based (5′- nuclease) assay

Table A-1 lists the 48 SNPs in the Control Pool.

Table A-1 48 SNPs in the SNPlex System Control Pool

Zip #[a]	Celera Discovery System™ ID (hCV #)	SNP Consortium ID (TSC #)	dbSNP ID (rs #)	Minor Allele Frequency				
				AfA M[b]	Cauc [c]	Chin [d]	Japn [e]	Location[f]
M5.1_001	hCV2058031	TSC0630913	rs1425151	0.23	0.26	0.28	0.28	Chr. 11_10,727,372
M5.1_002	hCV7547436	TSC0691193	rs1457947	0.39	0.42	0.49	0.44	Chr. 6_77,426,938
M5.1_003	hCV1901045	TSC0806211	rs1323881	0.29	0.20	0.38	0.37	Chr. 13_100,315,097
M5.1_004	hCV7536854	TSC0556240	rs1388276	0.46	0.33	0.34	0.45	Chr. 3_72,816,542
M5.1_005	hCV2597763	TSC0926934	rs1980408	0.12	0.23	0.48	0.44	Chr. 21_40,775,977
M5.1_006	hCV2059319	—	—	0.43	0.31	0.15	0.08	Chr. 11_19,245,581
M5.1_007	hCV8929459	TSC0339341	rs1035089	0.16	0.42	0.38	0.38	Chr. 16_11,016,789
M5.1_008	hCV2986015	TSC0295775	rs705681	0.21	0.45	0.22	0.22	Chr. 1_6,999,213
M5.1_009	hCV8720462	TSC0165692	rs893613	0.30	0.48	0.41	0.40	Chr. 15_87,994,667
M5.1_010	hCV349615	TSC0314577	rs992690	0.16	0.24	0.34	0.36	Chr. 12_16,550,429
M5.1_012	hCV8879897	—	—	0.43	0.46	0.44	0.45	Chr. 10_12,356,433
M5.1_013	hCV7505765	TSC0265430	rs961495	0.26	0.19	0.20	0.08	Chr. 14_100,278,705
M5.1_014	hCV1637791	TSC0016017	rs729673	0.45	0.40	0.24	0.20	Chr. 18_3,057,735
M5.1_015	hCV1691378	TSC0265475	rs1115261	0.48	0.46	0.18	0.28	Chr. 13_27,191,865

Table A-1 48 SNPs in the SNPlex System Control Pool *(continued)*

Zip #[a]	Celera Discovery System™ ID (hCV #)	SNP Consortium ID (TSC #)	dbSNP ID (rs #)	Minor Allele Frequency				
				AfAM[b]	Cauc[c]	Chin[d]	Japn[e]	Location[f]
M5.1_016	hCV3194437	TSC0301076	rs984071	0.20	0.29	0.17	0.17	Chr. 9_10,596,485
M5.1_017	hCV7571632	TSC0243654	rs927221	0.16	0.15	0.28	0.45	Chr. 14_66,832,202
M5.1_018	hCV7537265	TSC0129483	rs748573	0.32	0.22	0.33	0.33	Chr. 2_45,728,104
M5.1_019	hCV7589926	—	—	0.30	0.11	NA	NA	Chr. 9_3,834,392
M5.1_020	hCV8845932	TSC0324505	rs1156404	0.27	0.47	0.17	0.17	Chr. 6_70,690,465
M5.1_021	hCV2179737	—	—	0.33	0.20	0.43	0.31	Chr. 8_1,599,764
M5.1_022	hCV8792022	TSC0213380	rs879253	0.21	0.45	0.21	0.08	Chr. 5_14,827,607
M5.1_023	hCV2489240	TSC0098582	rs1016146	0.12	0.35	0.48	0.27	Chr. 6_35,446,932
M5.1_024	hCV2025116	TSC0757622	rs1507213	0.12	0.48	0.48	0.50	Chr. 12_85,457,089
M5.1_025	hCV1653240	TSC0136611	rs751340	0.42	0.37	0.24	0.18	Chr. 9_125,143,032
M5.1_026	hCV357822	TSC0783613	rs1520483	0.10	0.41	0.48	0.48	Chr. 3_46,329,443
M5.1_027	hCV8686971	TSC0465947	rs1570903	0.23	0.44	0.45	0.26	Chr. 13_94,986,498
M5.1_028	hCV706864	TSC0071873	rs288423	0.42	0.37	0.43	0.38	Chr. 15_95,755,875
M5.1_029	hCV3017144	TSC0084538	rs1007106	0.19	0.34	0.45	0.45	Chr. 8_124,612,406
M5.1_030	hCV9621778	TSC0318440	rs995178	0.41	0.49	0.49	0.45	Chr. 5_22,445,803
M5.1_031	hCV8747570	TSC0679949	rs1597695	0.35	0.39	0.50	0.44	Chr. 2_105,883,662
M5.1_032	hCV8862622	TSC0825772	rs1334334	0.15	0.24	0.27	0.17	Chr. 1_87,717,343
M5.1_033	hCV8946637	TSC0809047	rs794108	0.46	0.38	0.29	0.37	Chr. 6_164,517,177
M5.1_034	hCV1358402	TSC0463216	rs1569244	0.42	0.17	0.41	0.37	Chr. 6_164,517,144
M5.1_035	hCV7500677	TSC0296508	rs238196	0.12	0.10	0.28	0.30	Chr. 20_48,536,640
M5.1_036	hCV9589619	TSC0984433	rs1925643	0.42	0.30	0.42	0.42	Chr. 10_100,359,810
M5.1_037	hCV8921382	TSC0910879	rs1713423	0.42	0.50	0.31	0.41	Chr. 14_18,850,202
M5.1_038	hCV1688032	—	—	0.22	0.31	0.16	0.23	Chr. 1_184,612,692
M5.1_039	hCV9636350	—	—	0.16	0.37	0.36	0.37	Chr. 12_122,620,930
M5.1_041	hCV2962785	—	—	0.40	0.11	0.17	0.24	Chr. 22_25,219,892
M5.1_042	hCV2780152	TSC0851851	rs1861606	0.49	0.31	0.29	0.36	Chr. 12_22,349,685
M5.1_043	hCV2569743	TSC0430769	rs1548543	0.22	0.34	0.31	0.29	Chr. 19_14,933,236

Table A-1 48 SNPs in the SNPlex System Control Pool *(continued)*

Zip #[a]	Celera Discovery System™ ID (hCV #)	SNP Consortium ID (TSC #)	dbSNP ID (rs #)	Minor Allele Frequency				
				AfAM[b]	Cauc[c]	Chin[d]	Japn[e]	Location[f]
M5.1_044	hCV468629	TSC0463040	rs1569125	0.41	0.29	0.22	0.2	Chr. 2_236,572,816
M5.1_045	hCV1534177	—	—	0.22	0.20	0.27	0.29	Chr. 18_75,206,886
M5.1_046	hCV11164916	—	—	0.19	0.12	NA	NA	Chr. 7_21,876,339
M5.1_048	hCV2214945	TSC1086371	rs220860	0.25	0.22	0.22	0.22	Chr. 11_115,327,978
M5.1_049	hCV8847720	TSC0695101	rs1460239	0.29	0.40	0.19	0.29	Chr. 8_105,480,159
M5.1_050	hCV8777053	TSC0070807	rs954779	0.28	0.18	0.32	0.30	Chr. 9_36,409,531
M5.1_051	hCV7443819	TSC0267953	rs963014	0.48	0.40	0.08	0.11	Chr. 8_117,999,529

a. Indicates the correlation between a SNP and its corresponding ZipChute™ probe pair
b. African-American
c. Caucasian
d. Chinese
e. Japanese
f. Indicates chromosome and base position on the chromosome, as derived from the SNPbrowser™ software

About the Dried gDNA Plate

- Each gDNA plate contains 44 unique human DNAs of Caucasian origin.
- Each gDNA is plated at least twice in each quadrant of a 384-well microtiter plate to allow for the asessment of assay reproducibility (refer to the SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD).

  For all gDNAs, a consensus genotype was established using data from repeat SNPlex System assays using the control pool SNPs. These genotypes serve as a reference to calculate the accuracy of the system (refer to the SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD).
- gDNAs are plated into 384-well microtiter plates appropriate for use with the Applied Biosystems 3730/3730*xl* DNA Analyzer.
- The gDNA plate can be used in conjunction with custom human ligation probe pools. Comparing data achieved with the gDNA plate to data achieved with user-supplied DNA allows you to assess the quality of your DNAs.
- The proportion of female to male DNA is approximately 1:1.
- Users who prefer the 96-well protocol can resuspend the gDNA in one 384-well plate quadrant and transfer the contents to a 96-well plate (refer to "Using the SNPlex™ System Dried gDNA Plates" file on the CD).

Using the Control Pool

To use the SNPlex™ System Control Pool together with the dried gDNA plate, perform the SNPlex System assay as described in Chapter 3, substituting the SNPlex System Control Pool for the SNPlex™ Ligation Probe Pool. See the SNPlex™ Genotyping Dried gDNA Plate Control Pool System CD for information regarding the dispensing of the allelic ladder.

Expected Results

The following results can typically be expected when using the SNPlex System control pool together with the SNPlex System dried gDNA plate:

- Three genotype clusters should be observed for 45/48 SNPs. Exceptions are hcv7505765 (SNP #13), hcv7571632 (SNP #17), and hcv2962785 (SNP #41), which show only two clusters.
- The precision, or the measure of the reproducibility in genotype calls between identical gDNA samples, should be 99.7% or better.
- The accuracy, or the measure of the genotype data achieved with the SNPlex System against reference genotypes, should be 99.5% or better.
- The call rate, or the measure of the number of genotypes made relative to all possible calls, should be 95% or better.

Note: If you use your own gDNA plate together with the SNPlex System control pool, the signal strength and cluster angle you observe for one SNP should be similar to what you observe with the dried gDNA plate.

Figure A-1 shows the cluster plots for each of the 48 SNPs. (Cluster plots are numbered. Note that numbers 40 and 47 are not associated with a SNP in this figure.)

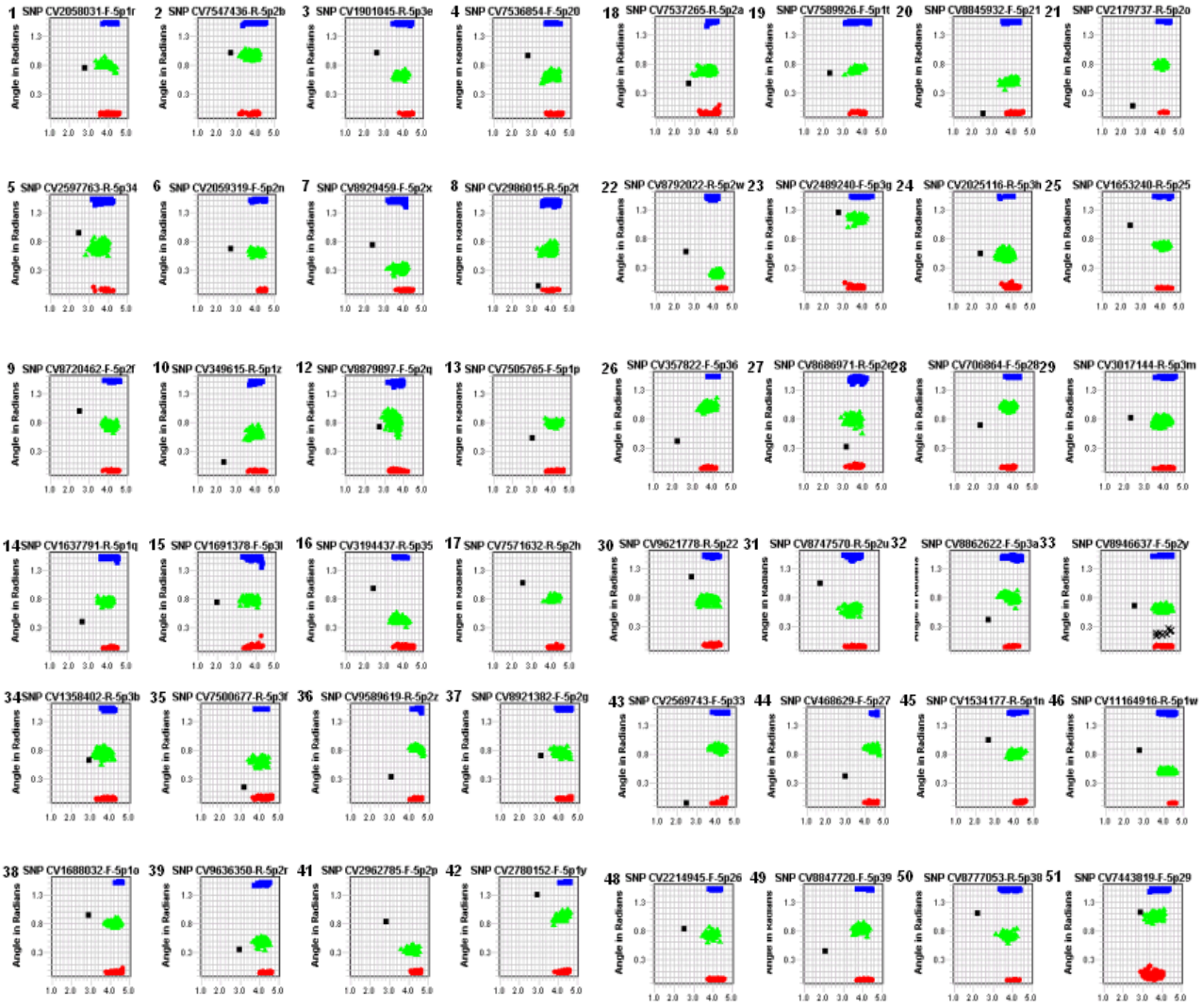


Figure A-1 Cluster plots of the 48 SNPs in the Control Pool

GeneMapper Software v3.7: Analysis Methods for SNPlex System Assays

B

This chapter covers:

Analysis Methods for SNPlex System Assays

Overview

An analysis method is a set of algorithm parameters that you apply to samples in a project before analyzing data. The GeneMapper® software v3.7 uses analysis methods for peak detection, allele calling, peak quality quantification, and quality of results reports.

GeneMapper software v3.7 includes two default methods for analyzing SNPlex™ system data:

- **SNPlex_Rules_3730** – Uses the Rules clustering algorithm to calculate the SNP quality according to a set of custom rules.
- **SNPlex_Model_3730** – Uses the Model clustering algorithm, which compares the behavior of alleles to an ideal (or "model"), to calculate the SNP quality.

Selecting a Clustering Algorithm

The parameters of an analysis method are specified in the tabs of the Analysis Method Editor. The software online help provides detailed information about each of these parameters. Note that analysis parameters for SNPlex System applications are different from those for other applications (such as AFLP® kits or microsatellites).

To view the descriptions for SNPlex System analysis parameters in the software online help, click the **Workflows** tab. Under Applications-specific Workflows, click **SNPlex Analysis Workflow**, then click **Creating Analysis Methods**.

Of all the parameters, the clustering algorithm determines how the analysis method analyzes data. The default analysis methods for SNPlex System data are named after the clustering algorithm used in the method.

The following table explains how to decide which method to use for your data.

Use the SNPlex_Model_3730 Method ...	Use the SNPlex_Rules_3730 Method ...
For high-throughput experiments with 40 to 94 samples per run. • Samples must contain DNA. Positive controls are included in the sample count. • Samples do not include the no template control (NTC) and allelic ladder. • If ≤24 samples are included in a run, the GeneMapper software will not analyze the data using the model-based method. Note that to use the Model algorithm, probe pools must contain at least 18 SNPs.	• For low-frequency SNPs. • When you want to apply the filters, such as the Hardy-Weinberg filter, used by the Rules algorithm. (Refer to "Modifying Values of Individual Rules (Rules Method Only)" on page B-8 for more information.) • When you have fewer than 40 samples.

Terms Used in Clustering Analysis

The following table defines commonly used terms in clustering analysis. For more information about these concepts, refer to the GeneMapper™ Software v3.7 online help.

Term	Meaning
Confidence Value (CV)	Indicates how confident the software is that a call it has made for a particular point is correct.
Process (Component-based) Quality Values (PQV)	Reported by the software to aid in finding and fixing problems in sample preparation and analysis.
Quality Flag	Based on the value of the PQV. For example, if SQ = 1, the quality flag for SQ will be green (pass).
Sizing Quality (SQ)	If the sizing quality of a SNP falls within the low quality range, the software does not process the sample any further. Note that SQ values for SNPlex System experiments are higher than that for other applications.
Genotype Quality (GQ)	Quality flag whose value determines whether the software passes or fails a genotype. The calculated value is based on allele quality values (AQ) of the two alleles in the SNP and the weighted values of each PQV. The GeneMapper software online help provides detailed information about calculating the GQ. For the Rules method, the GQ is zeroed out if GQs fall below the CV. For the Model method, the GQ is not set to zero if the value falls below the CV.
Well Quality (WQ)	Quality flag that represents the mean confidence value (CV) for all SNPs within a well. The software fails wells whose CVs fall below the pass range specified for WQ.
SNP Quality (SNPQ)	Value serving as a preliminary indicator of the quality of an analyzed SNP. For rules-based methods, 1 indicates a passing SNP; 0 indicates a failing SNP. For model-based methods, the SNPQ is reported as a value between 0 and 1.

The following sections describe the default methods for analyzing SNPlex System data.

SNPlex_Rules_3730

How the Rules Algorithm Works

Figure B-1 summarizes how the Rules algorithm calculates the SNP quality.

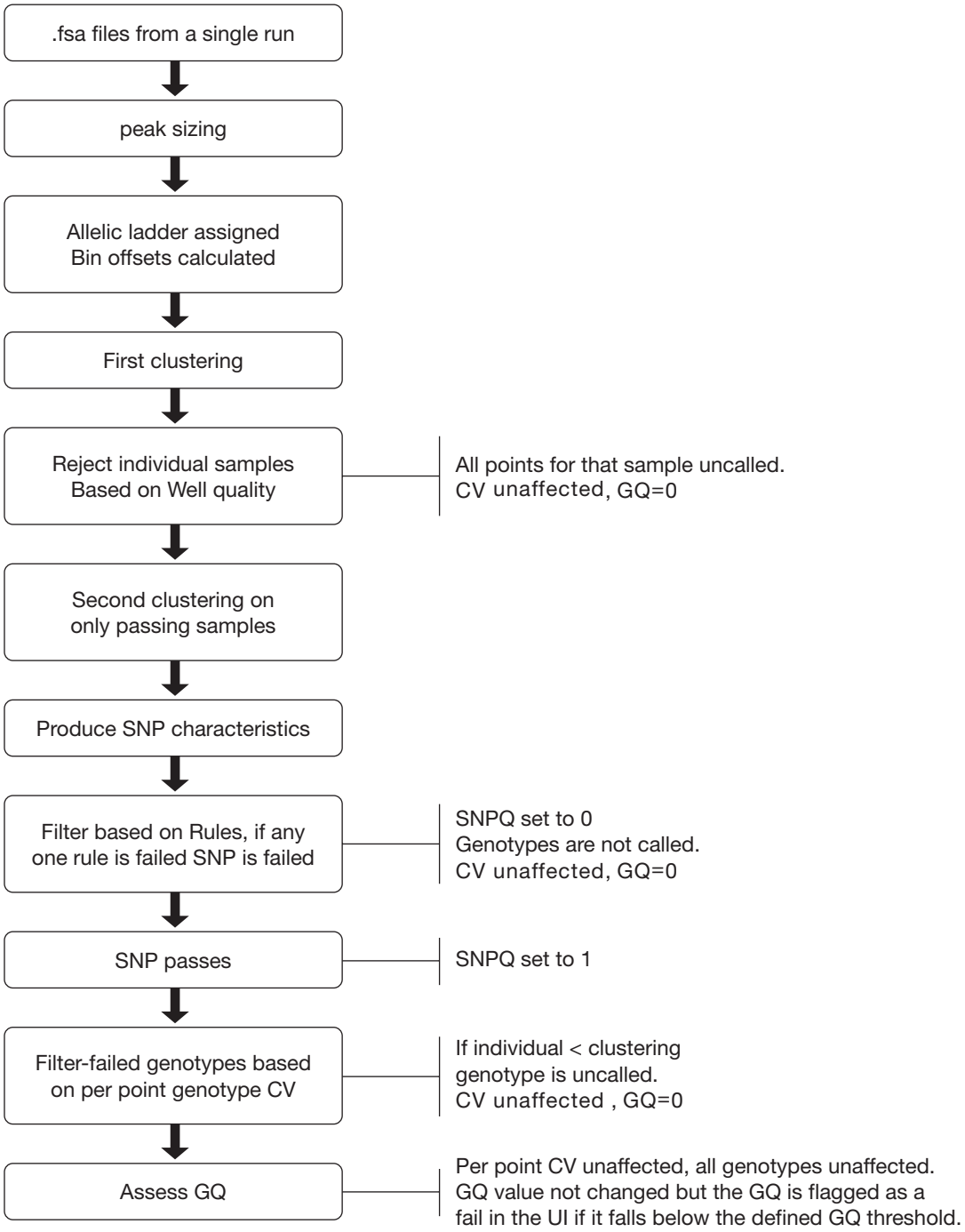


Figure B-1 Summary of the Rules Algorithm

Settings of the SNPlex_Rules_3730 Method

Table B-1 lists the default settings of the SNPlex_Rules_3730.

Table B-1 Default settings for the SNPlex_Rules_3730

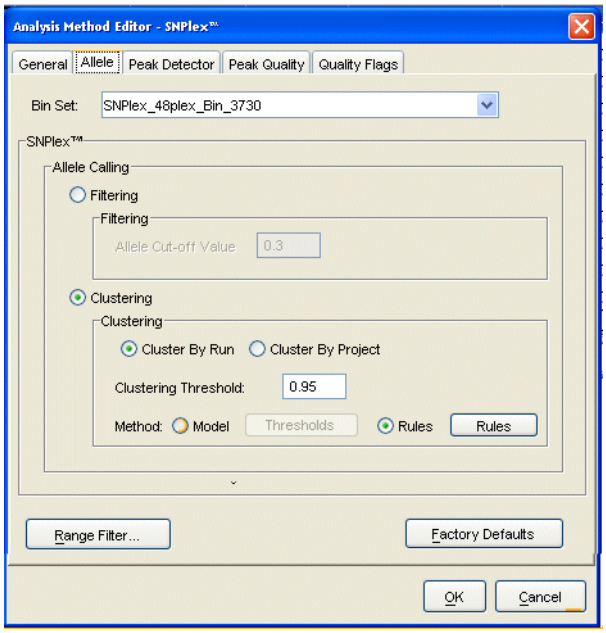
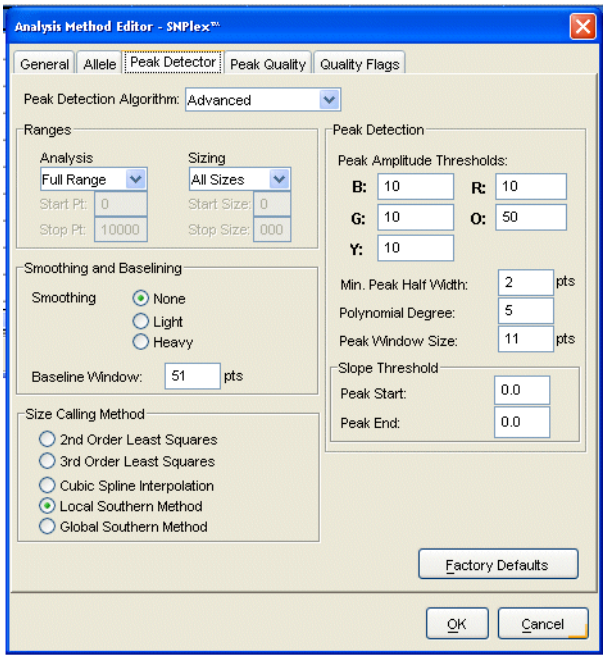
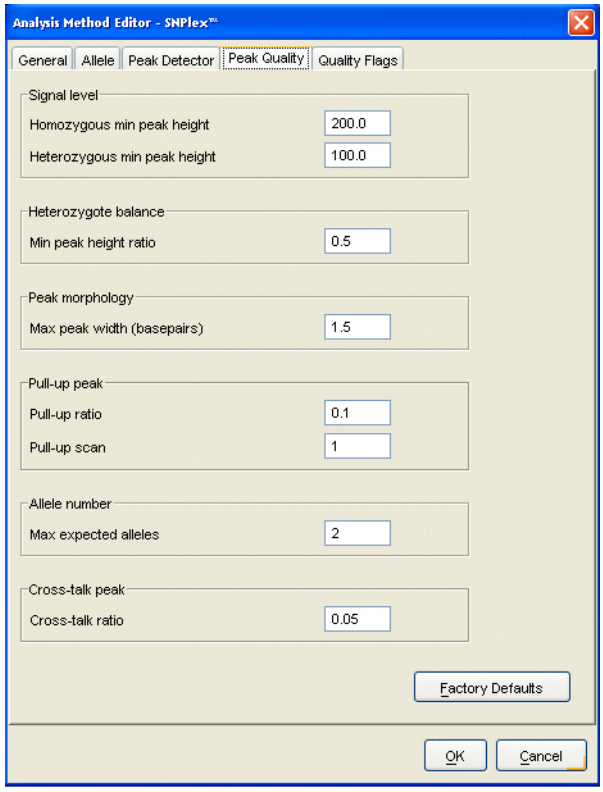
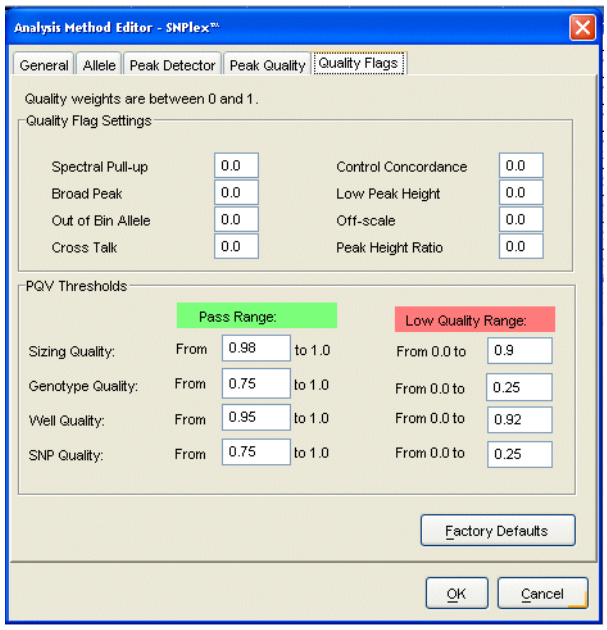
Tab	Default Settings	Modifying the Default Settings
General	Name: **SNPlex_Rules_3730**	—
Allele		You can modify the following settings in the Allele tab: • Bin Set – Make sure that the bin set matches the panel used for the analysis. For SNPlex System experiments, the bin set is always **SNPlex_48plex_Bin_3730**. • Allele Calling Method – Refer to "Selecting an Allele Calling Method" on page B-7 for information about selecting an allele calling method. – Clustering – If selected, you can set the clustering threshold and specify how data is clustered. You can also modify values of the rules that the algorithm uses to calculate SNP quality. Refer to "Modifying the Clustering Parameters" on page B-7 for more information.
Peak Detector		Refer to the online help for a detailed description of the parameters in the Peak Detector tab. (Search on the topic "SNPlex Peak Detector".) • For SNPlex System experiments, the Peak Detection Algorithm must be **Advanced**. • The Peak Amplitude Thresholds should be low (default is 10 for B,G, Y, and R and 50 for O). – If set below 10, background noise may lengthen and interfere with analysis. – If set above 10, clustering is affected.

Table B-1 Default settings for the SNPlex_Rules_3730

Tab	Default Settings	Modifying the Default Settings
Peak Quality		Refer to the online help for a detailed description of the parameters in the Peak Quality tab. (Search on the topic “SNPlex Peak Quality”.) Although the software calculates values for the parameters in this tab, the peak quality parameters do not affect the Genotype Quality because the Quality Flags are set to 0.
Quality Flags		Refer to the online help for a detailed description of the parameters in the Peak Quality tab. (Search on the topic “SNPlex Quality Flag”.) For SNPlex System default analysis methods, all quality flags are set to 0. Consequently, the software does not use these flags to pass or fail a SNP. However, the PQVs are used to calculate the genotype quality, as shown in Figure B-1 on page B-4.

Selecting an Allele Calling Method

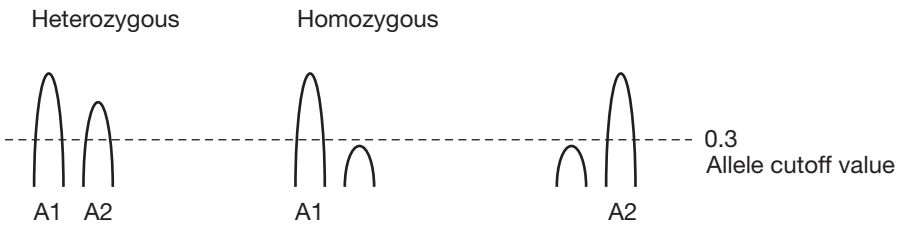
GeneMapper Software v3.7 provides two allele calling methods:

- Filtering – The filtering method allows you to analyze sample data based solely on peak height, without clustering analysis.
- Clustering – This method makes calls based on a minimum confidence value for a sample in a particular cluster. Most SNPlex System data is analyzed using this method.

Modifying the Allele Cut-off Value

If you select the Filtering method, you can specify an Allele Cutoff Value, which is the value below which a peak is recognized as an allele. The allele cutoff value is the percentage of the larger peak's height required for the smaller peak to be called as an allele.

IMPORTANT! Applied Biosystems does not recommend using this method.



Modifying the Clustering Parameters

If you select the Clustering method for allele calling, you can

- Specify how data is clustered
- Set the clustering threshold
- Modify values of the rules that the algorithm uses to calculate SNP quality

Specifying How Data is Clustered

- Select **Cluster By Run** for the software to analyze the samples in one run independently from other runs. Most SNPlex System data is clustered according to this method.

  IMPORTANT! If you are using the Model method, you must select Cluster By Run.

- Select **Cluster By Project** for the software to analyze all of the selected samples in a project, regardless of whether the samples were run at the same time. If you select Cluster By Project, make sure that you select the SNP set before analyzing your project.

  For example, if you have 10 runs out of 96 in which a single data point is shown as Heterozygote 2 (Het 2), by clustering the 10 runs in the project, you obtain 10 points displayed as Het 2. Consequently, it is easier to determine if the call is accurate or if it is an experimental artifact.

Note: Regardless of the clustering method that you select, bin offsets are calculated by run (that is, by grouping all samples in a folder into a single run).

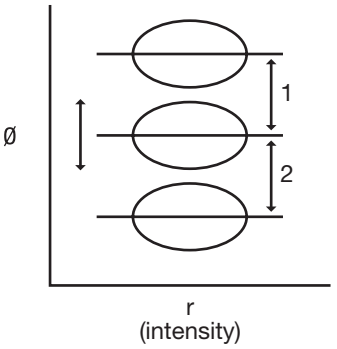
Setting the Clustering Threshold

The clustering threshold value is the minimum confidence value of the sample in a cluster.

- For the Rules method, if the confidence value for an individual data point in a particular cluster is >0.95, the point is labeled with the genotype for that cluster. If the CV falls below the threshold, the genotype is zeroed out.
- For the Model method, the genotype for the data point is always displayed, whether or not the sample passes the threshold. (An exception is when a sample is removed from analysis, none of its data points will have a CV.)

Modifying Values of Individual Rules (Rules Method Only)

An important point to remember about the Rules method is that the SNP needs to fail only one of the six rules for the software to fail the SNP. The following table lists the six rules according to which SNPs are assessed.

Rule	Description
Number of clusters must be no less than	If the software detects fewer clusters than specified in this rule, the SNP is failed.
Sigma separation must be no less than	To determine this value, the software measures cluster resolution ("fuziness") and distance between the two clusters. It reports a single value that encompasses both cluster width and separation.  Applied Biosystems recommends that you do not set this value to < 6 because doing so decreases the accuracy. Raising the value causes a slight increase in accuracy, but also causes more SNPs to fail.
Hardy-Weinberg p-value must be no less than	If set to n, n% of the time, a valid SNP is failed. For example, if set to 0.01, 1% of valid SNPs are failed.
Angle between clusters must be no larger than	Applies only to SNPs with only two clusters. In these cases, this rule ensures that the software does not call two homozygotes for a single SNP.
Call rate must be no less than	Percentage of genotypes that must be called for a SNP in order for the SNP to pass. For example, if Call Rate is 0.8, 80% of the genotypes for a SNP must be called in order for the SNP to pass.
Signal median value must be no less than	Median value; 50% of the signals for a SNP fall below this value. If you modify this value, Applied Biosystems recommends that you raise it. Do not lower the median value.

Thresholds for the Model Algorithm

IMPORTANT! Applied Biosystems recommends that you do not modify these settings.

The Model algorithm rejects samples according to the following threshold values:

- Well signal threshold – Represents the mean signal (from both alleles) of a well. It is the sum of all the peaks, divided by the number of SNPs.
- Well template threshold – Represents how far the well's behavior is from an ideal well (0.5). The software rejects wells that have values that fall below this threshold.

SNPlex_Model_3730

How the Model Algorithm Works

Figure B-2 summarizes how the Model algorithm calculates the SNP quality.

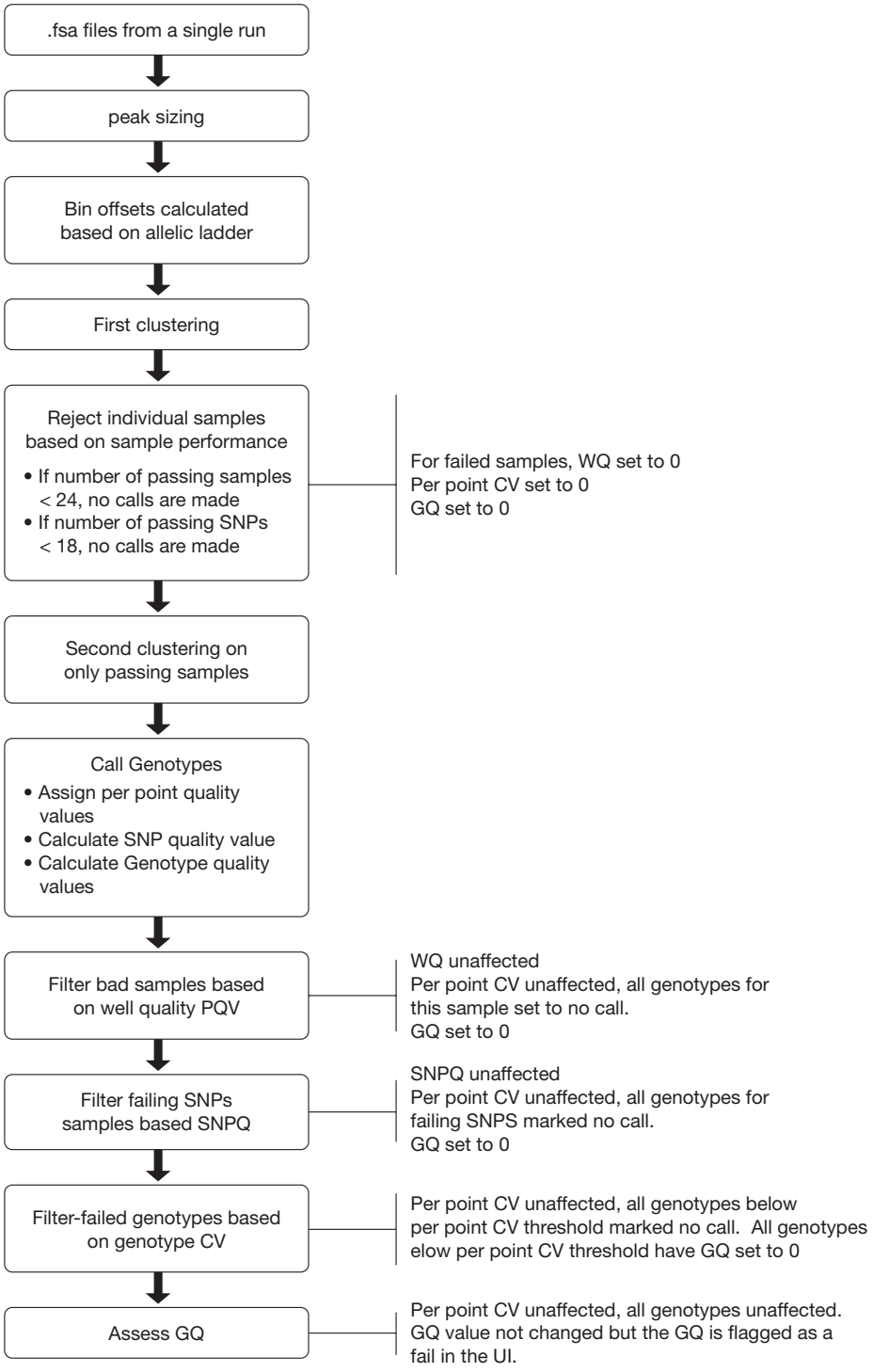
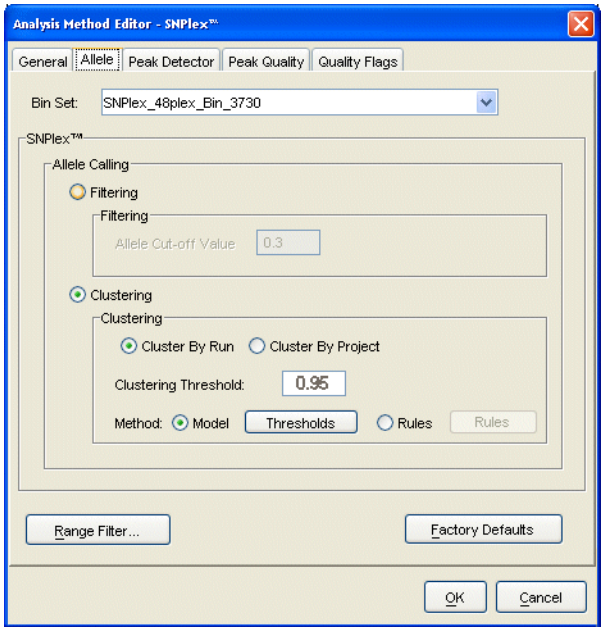
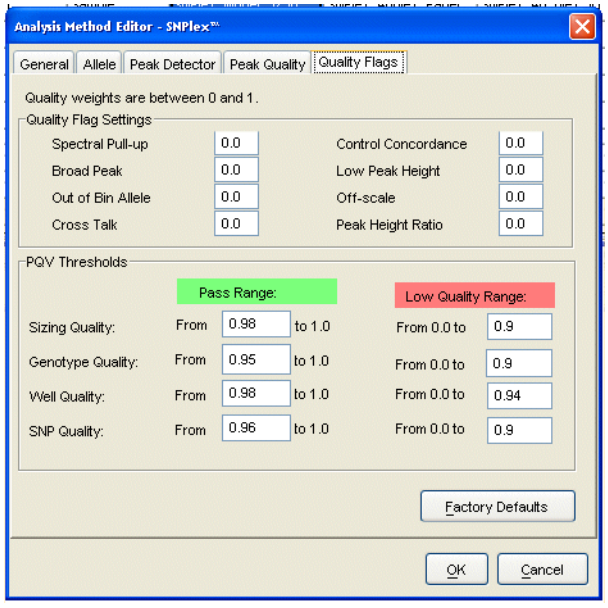


Figure B-2 Summary of the Model Algorithm

Settings of the SNPlex_Model_3730 Method

Table B-2 lists the default settings of the SNPlex_Model_3730.

Table B-2 Default settings for the SNPlex_Model_3730

Tab	Default Settings	Modifying the Default Settings
General	Name: **SNPlex_Model_3730**	—
Allele		You can modify the following settings in the Allele tab: • Bin Set – Make sure that the bin set matches the panel used for the analysis. For SNPlex System experiments, the bin set is always **SNPlex_48plex_Bin_3730**. • Allele Calling Method – Refer to "Selecting an Allele Calling Method" on page B-7 for information about selecting an allele calling method. – Clustering – If selected, you can set the clustering threshold and specify how data is clustered. You cannot use the Cluster By Project option. Refer to "Modifying the Clustering Parameters" on page B-7 for more information. **IMPORTANT!** Applied Biosystems recommends that you accept the default settings for the Model threshold values.
Peak Detector	Same as the settings for the Rules method. Refer to "Peak Detector" on page B-5.	Refer to "Peak Detector" on page B-5.
Peak Quality	Same as the settings for the Rules method. Refer to "Peak Quality" on page B-6.	Refer to "Peak Quality" on page B-6.
Quality Flags		Refer to the online help for a detailed description of the parameters in the Peak Quality tab. (Search on the topic "SNPlex Quality Flag".) For SNPlex System default analysis methods, all quality flags are set to 0. Consequently, the software does not use these flags to pass or fail a SNP. However, the PQVs are used to calculate the genotype quality, as shown in Figure B-2 on page B-10.

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